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REGULAR PAPER – TAXONOMY/PHYLOGENETICS/EVOLUTIONARY BIOLOGY

Phylogeny and biogeography of *Sagittaria* (Alismataceae) revisited: evidence for cryptic diversity and colonization out of South America

Yu Ito[1] · **Norio Tanaka**[2] · **Brian R. Keener**[3] · **Samuli Lehtonen**[4]

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Abstract

Sagittaria is a genus of ca. 40 species in the aquatic plant family Alismataceae with a nearly global distribution, and a center of diversity in the New World. Two thirds of the known species are native to the Americas, while only a few species are distributed in Africa, Asia and Europe. A previous biogeographic analysis of the genus suggested an African origin for the genus with subsequent dispersal to North America and then to East Asia. Here we expanded the taxon sampling with a focus on the New World taxa and applied species delimitation and biogeographic analyses to revise the knowledge of the phylogeny and evolution of the genus. We obtained largely similar topologies from the chloroplast DNA and nuclear DNA (ITS) data sets. The 74 accessions sampled for our analyses were delimited into 29 species and several cryptic taxa were revealed in widely distributed species. Biogeographic analysis supported basal diversification in South America and subsequent colonization to North America and Asia.

Keywords Alismatales · Aquatic plants · Biogeography · Molecular phylogeny · Species delimitation · South America · STACEY

Introduction

Aquatic plants are found in aquatic environments throughout the world and tend to show relatively low species diversity and widespread distributions (de Candolle 1855; Sculthorpe 1967). Phenotypic plasticity is often remarkable in aquatic plants and in many cases leads to difficulties in taxonomic and systematic studies (e.g., Kaplan 2002). Recent molecular phylogenetic studies have revealed cases of widespread aquatic plants in which the species diversity had been under-estimated. For example, Ito et al. (2010, 2013, 2015) collected a cosmopolitan species, *Ruppia maritima* L. (Ruppiaceae), across the world and discerned a species complex of seven taxonomic entities as well as found a cryptic species that was described as a new species from South Africa. Ito et al. (2019) and Li et al. (2020) collected and analyzed multiple samples of *Ottelia alismoides* (L.) Pers. across Asia and *O. ulvifolia* (Planch.) Walp. in Africa (Hydrocharitaceae), respectively, and revealed cryptic diversity within these widespread species.

Sagittaria L. is a genus of an aquatic family Alismataceae that contains ca. 40 species in the world. Some species show great phenotypic plasticity depending on the aquatic environment (Haynes and Hellquist 2000). Some *Sagittaria* species are also widely distributed, such as *S. graminea* Michx., *S. lancifolia* L., *S. latifolia* Willd., and *S. montevidensis* Cham. and Schltdl in the Americas (Crow and Hellquist 2000; Haynes and Hellquist 2000; Haynes and Holm-Nielsen 1994, 1995; Holm-Nielsen and Haynes 1986; Proctor 2005), *S. guayanensis* Kunth in the pantropics (Haynes and Hellquist 2000; Wang et al. 2010), and *S. natans* Pall. in Eurasia (Dandy 1980; Tanaka 2015; Wang et al. 2010). It is possible that these wide ranging *Sagittaria*

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✉ Yu Ito
yu.ito@pharm.setsunan.ac.jp

[1] Faculty of Pharmaceutical Sciences, Setsunan University, 45-1 Nagaotoge-cho, Hirakata, Osaka 573-0101, Japan

[2] Tsukuba Botanical Garden, National Museum of Nature and Science, 4-1-1, Amakubo, Tsukuba, Ibaraki 305-0005, Japan

[3] University of West Alabama, Livingston, AL 35470, USA

[4] Biodiversity Unit, University of Turku, 20014 Turku, Finland

species may actually represent species complexes of previously unnoticed diversity.

Biogeography of widely distributed aquatic plants has interested botanists for decades (e.g., Les et al. 2003). A previous study on the phylogeny and biogeography of Alismataceae included 11 *Sagittaria* accessions representing ten species from Asia and North America and inferred North America and Africa as the ancestral area for the genus (Chen et al. 2012). However, the inference seems preliminary because only a few American species of *Sagittaria* were sampled, despite the Americas being the center of diversity for the genus with ca. 30 species (Haynes and Hellquist 2000; Holm-Nielsen and Haynes 1986; Keener 2005). In order to address the historical biogeography more accurately, a follow-up analysis based on a taxonomically more comprehensively sampled molecular phylogenetic tree is needed.

In the present study we aim to revisit the phylogeny of *Sagittaria* by expanding the previously limited taxonomic sampling with a better representation of the species from the Americas. In particular, we collected multiple samples from the widely distributed species mentioned above and applied a species delimitation analysis (STACEY: species tree estimation using DNA sequences from multiple loci; Jones 2017) to test the presence of cryptic species. We also use the new densely sampled molecular phylogeny to revise the biogeographic history of the genus.

Materials and methods

Taxon sampling

Samples of *Sagittaria* were collected in the field or obtained from herbarium specimens (Fig. 1; Table 1). The sampled specimens were identified based on the taxonomic treatments of Bogin (1955), Crow and Hellquist (2000), Haynes and Hellquist (2000), and Keener (2005) for the North American species; Holm-Nielsen and Haynes (1986), Haynes and Holm-Nielsen (1994, 1995), and Lehtonen (2018) for the South American species; Wang et al. (2010) and Tanaka (2015) for the Asian species; and Dandy (1980) for European species. Infraspecific taxa in *S. graminea* and *S. montevidensis* recognized by Haynes and Hellquist (2000) were treated as species following Keener (2005), namely *S. chapmanii* (J.G. Sm.) C. Mohr and *S. calycina* Engelm. Identifications to infraspecific taxa in *S. guayanensis* followed Haynes and Hellquist (2000) and Wang et al. (2010). A single specimen from Ohio, USA, was tentatively identified as *S.* cf. *australis* (J.G. Sm.) Small (OS432686) based on the available digital image of the specimen. Our taxon sampling covered 28 recognized species and two subspecies (Fig. 1; Table 1). *Limnophyton* Miq. and *Caldesia* Parl., representatives of two closely

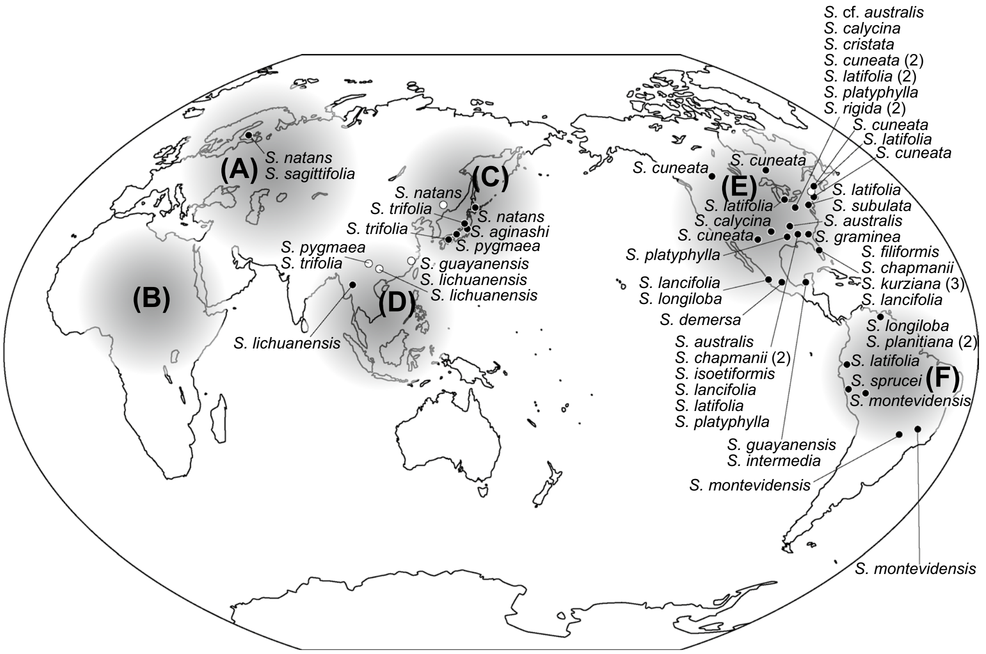

Fig. 1 Map of sampling localities of *Sagittaria* species included in this study. Shaded areas correspond to the seven biogeographic areas delimited for the biogeographic analysis. Samples collected in this study are shown with closed dots and those from Chen et al. (2012) with open dots. The number of specimens per species per area is shown in parentheses. Only the native accessions are shown here; the samples of garden origin (one each for *S. graminea*, *S. montevidensis*, *S. platyphylla*, *S. potamogetifolia*, and *S. trifolia*) are not shown. The area codes are used for biogeographic analysis (Fig. 4)

Table 1 Sample localities, GenBank accessions numbers for *Sagittaria* species and their outgroup taxa

No	Taxon	Locality	Date	voucher	*matK*	*psbA*	*rbcL*	ITS
n/a	*Caldesia grandis*	n/a	n/a	n/a	JF781068	JF975417	JF781043	n/a
n/a	*Limnophyton angolense*	n/a	n/a	n/a	JF781076	JF975427	JF781049	JF780992
TD5915	*Sagittaria aginashi*	Japan: Ibaraki, Inashiki	20 Sep 2014	Tanaka, N. TD5915 (TNS)	LC541374	LC541612	LC541671	LC541313
OS432686	*Sagittaria* cf. *australis*	USA: Ohio, Vinton	14 Sep 2007	Gardner, R.L. 5639 (OS432686)	LC541390	LC541629	LC541686	LC541331
BRK9226	*Sagittaria australis*	USA: Alabama, Jefferson	13 Sep 2015	Keener, B.R. #9226 (UWAL0030187)	LC541415	LC541658	LC541713	LC541360
BRK1928	*Sagittaria australis*	USA: Kentucky, Muhlenberg	02 Sep 2000	Keener, B.R. 1928 (UWAL)	LC541416	LC541659	LC541714	LC541361
#8842	*Sagittaria calycina*	USA: Missouri, New Madrid	29 Sep 1994	Horn, C.N. #8842 (GH)	n/a	LC541613	n/a	LC541314
OS432801	*Sagittaria calycina*	USA: Ohio, Ottawa	27 Jul 2005	Arbour, T.P. #163 (OS432801)	n/a	LC541630	n/a	LC541332
GH01632424	*Sagittaria chapmanii*	USA: Florida, Wakulla	01 May 1973	Slaney, T. #215 (GH01632424)	LC541381	LC541621	n/a	LC541322
Diamond18908	*Sagittaria chapmanii*	USA: Alabama, Houston	10 Apr 2008	Diamond #18908 (UWAL0003459)	n/a	LC541660	LC541715	LC541362
Diamond26315	*Sagittaria chapmanii*	USA: Alabama, Houston	07 May 2015	Diamond #26315 (UWAL0032142)	LC541417	LC541661	LC541716	LC541363
OS399371	*Sagittaria cristata*	USA: Ohio, Lucas	09 Jul 1996	Schneider, G. 1996:63 (OS399371)	LC541382	LC541622	LC541678	LC541323
No. 89-326-M	*Sagittaria cuneata*	Canada: Quebec, Island of Orleans	29 Aug 1989	Garneau, M. 89-326-M (GH)	n/a	LC541615	n/a	LC541316
#4133	*Sagittaria cuneata*	USA: Massachusetts, Hanson	11 Sep 1997	Zebryk, T.M. #4133 (NEBC01080295)	LC541376	LC541616	LC541673	LC541317
GH16627	*Sagittaria cuneata*	USA: New Mexico, San Juan	19 Jul 2001	Hellquist, C.B. 16627 & Reeves, T. (GH)	LC541377	LC541617	LC541674	LC541318
OS401782	*Sagittaria cuneata*	USA: Ohio, Lucas	25 Jun 1998	Schneider, G. 1998:92 (OS401782)	LC541379	LC541619	LC541676	LC541320
YI1670	*Sagittaria cuneata*	Canada: Alberta, Southern Alberta	20 Jul 2011	Ito, Y. YI1670 (TNS)	LC541380	LC541620	LC541677	LC541321
177225	*Sagittaria cuneata*	USA: Maine, Aroostook	20 Aug 1986	Hill, S.R. 17225 (GH)	LC541384	LC541623	LC541680	LC541325
OS432642	*Sagittaria cuneata*	USA: Ohio, Ottawa	14 Jul 2009	Gardner, R.L. 6427 (OS432642)	LC541389	LC541628	LC541685	LC541330
SL720	*Sagittaria demersa*	Mexico: Hidalgo	19 Oct 2005	Ramirez-Garcia 720 (TUR)	LC541404	LC541646	LC541701	LC541349
BRK1926	*Sagittaria filiformis*	USA: Florida, Leon	18 Aug 2000	Keener, B.R. #1926 (UWAL)	LC541418	LC541662	LC541717	LC541364
GH20514	*Sagittaria graminea*	USA: Georgia, Appling	25 May 1999	Nelson, J.B. 20514 (GH)	LC541383	n/a	LC541679	LC541324
BKR1957	*Sagittaria graminea*	USA: Florida, Wakulla	07 Oct 2000	Keener, B.R. #1957 (UWAL)	LC541419	LC541663	LC541718	LC541365
Chen	*Sagittaria guayanensis* subsp. *lappula*	China: Fujian, Wuyishan	n/a	n/a	JF781081	JF975434	JF781054	JF780968
SL406	*Sagittaria guayanensis* subsp. *guayanensis*	Mexico: Campeche	30 Aug 2005	Lehtonen, S. & Ramirez-Garcia 406 (TUR)	LC541405	LC541647	LC541702	LC541350

Table 1 (continued)

No	Taxon	Locality	Date	voucher	*matK*	*psbA*	*rbcL*	ITS
SL397	*Sagittaria intermedia*	Mexico: Ciudad del Carmen	29 Aug 2005	Lehtonen, S. & Ramirez-Garcia 397 (TUR)	LC541403	LC541645	LC541700	LC541348
BRK1949	*Sagittaria isoetiformis*	USA: Alabama, Covington	07 Oct 2000	Keener, B.R. #1949 (UWAL)	LC541420	LC541664	LC541719	LC541366
BRK1923	*Sagittaria kurziana*	USA: Florida, Wakulla	17 Aug 2000	Keener, B.R. #1923 (UWAL)	LC541421	LC541665	LC541720	LC541367
BRK1924	*Sagittaria kurziana*	USA: Florida, Wakulla	17 Aug 2000	Keener, B.R. #1924 (UWAL)	LC541422	LC541666	LC541721	LC541368
BRK1929	*Sagittaria kurziana*	USA: Florida, Alachua	18 Aug 2000	Keener, B.R. #1929-1 (UWAL)	LC541423	LC541667	LC541722	LC541369
SL407	*Sagittaria lancifolia*	Mexico: Nayarit	03 Sep 2005	Lehtonen, S. & Ramirez-Garcia 407 (TUR)	LC541406	LC541648	LC541703	LC541351
BRK1955	*Sagittaria lancifolia*	USA: Florida, Wakulla	07 Oct 2000	Keener, B.R. #1955 (UWAL)	LC541425	n/a	LC541724	LC541371
BRK1944	*Sagittaria lancifolia*	USA: Alabama, Mobile	29 Sep 2000	Keener, B.R. #1944 (UWAL)	LC541424	LC541668	LC541723	LC541370
OS365039	*Sagittaria latifolia*	USA: Ohio, Erie	08 Sep 1993	Cusick. A.W. #31,295 (OS365039)	LC541378	LC541618	LC541675	LC541319
No. 2615	*Sagittaria latifolia*	USA: Michigan, Marquette	05 Aug 1988	Henson, D. 2615 (GH)	LC541385	LC541624	LC541681	LC541326
850	*Sagittaria latifolia*	USA: New York, Jefferson	21 Jul 1992	Popolizio, C.A. & Close, B. 850 (GH)	LC541386	LC541625	LC541682	LC541327
Chen	*Sagittaria latifolia*	USA: New Hampshire, Dublin	n/a	n/a	JF781087	JF975441	n/a	JF780975
OS175190	*Sagittaria latifolia*	Canada: Ontario, Kent	24 Sep 1996	Cusick, A.W. #33,466 (OS175190)	LC541387	LC541626	LC541683	LC541328
OS422108	*Sagittaria latifolia*	USA: Ohio, Lucas	02 Sep 2005	Gardner, R.L. 5148 (OS422108)	LC541388	LC541627	LC541684	LC541329
GH1037	*Sagittaria latifolia*	USA: Alabama, Dallas	18 Jun 1991	Wen, J. 1037 (GH)	LC541398	LC541639	LC541694	LC541342
#562	*Sagittaria latifolia*	USA: Massachusetts, Hyannis	05 Aug 1980	Sorrie, B.A. #562 (NEBC01080383)	LC541399	LC541640	LC541695	LC541343
SL489	*Sagittaria latifolia*	Ecuador: Los Rios	25 Oct 2005	Lehtonen, S. & Navarrete 489 (TUR)	LC541407	LC541649	LC541704	LC541352
SL411	*Sagittaria latifolia*	Mexico: Nayarit	04 Sep 2005	Lehtonen, S. & Ramirez-Garcia 411 (TUR)	LC541411	LC541653	LC541708	LC541356
BRK1931	*Sagittaria latifolia*	USA: Alabama, Hale	05 Sep 2000	Keener, B.R. #1931 (UWAL)	LC541426	LC541669	LC541725	LC541372
Chen	*Sagittaria lichuanensis*	China: Guizhou, Pingba	n/a	n/a	JF781079	JF975432	JF781052	JF780966
Chen	*Sagittaria lichuanensis*	China: Fujian, Wuyishan	n/a	n/a	JF781080	JF975433	JF781053	JF780967
TD5581	*Sagittaria lichuanensis*	Myanmar: Sagaing	10 Dec 2016	Tanaka, N. et al. MY1871 (TNS)	LC541392	LC541633	LC541689	LC541335
SL410	*Sagittaria longiloba*	Mexico: Nayarit	04 Sep 2005	Lehtonen, S. & Ramirez-Garcia 410 (TUR)	LC541408	LC541650	LC541705	LC541353
SL466	*Sagittaria longiloba*	Venezuela: Falcon	07 Oct 2005	Lehtonen, S. 466 (TUR)	LC541409	LC541651	n/a	LC541354

Table 1 (continued)

No	Taxon	Locality	Date	voucher	*matK*	*psbA*	*rbcL*	ITS
Chen	*Sagittaria montevidensis*	Garden-origin	n/a	n/a	JF781086	JF975440	JF781060	JF780974
SL180	*Sagittaria montevidensis*	Bolivia: Beni	02 Sep 2003	Lehtonen, S. 180 (TUR)	EF088101	n/a	LC541706	EF088052
TD5637	*Sagittaria montevidensis*	Garden-origin	06 Jul 2017	Tanaka, N. TD5637 (TNS)	LC541391	LC541631	LC541687	LC541333
YI2030	*Sagittaria montevidensis*	Argentina: Entre Rios, Diamante	09 Dec 2014	Ito, Y. YI2030 (TNS)	n/a	LC541632	LC541688	LC541334
SL258	*Sagittaria montevidensis*	Paraguay: Misiones	01 Oct 2004	Lehtonen, S. & Burguez 258 (TUR)	LC541410	LC541652	LC541707	LC541355
Chen	*Sagittaria natans*	China: Heilongjiang, Qiqihaer	n/a	n/a	JF781082	JF975435	JF781055	JF780969
TD5633	*Sagittaria natans*	Japan: Hokkaido	16 Jun 2017	Tanaka TD5633 (TNS)	LC541393	LC541634	LC541690	LC541336
TUR594769	*Sagittaria natans*	Finland: Keski-Pohjanmaa	04 Jul 2010	Issakainen, J. s.n. (TUR594769)	LC541412	LC541654	LC541709	LC541357
SL428	*Sagittaria planitiana*	Venezuela: Cojedes	22 Sep 2005	Lehtonen, S. & Pacheco 428 (TUR)	EF088135	LC541655	LC541710	EF088088
SL448	*Sagittaria planitiana*	Venezuela: Guarico	01 Oct 2005	Lehtonen, S. & Pacheco 448 (TUR)	LC541413	LC541656	LC541711	LC541358
Chen[a]	*Sagittaria* cf. *platyphylla*	Garden-origin	n/a	n/a	JF781084	JF975437	JF781057	JF780971
YI2297	*Sagittaria platyphylla*	Malaysia: Rimba Ilmu	08 Aug 2016	Ito, Y. YI2297 (TNS)	LC541375	LC541614	LC541672	LC541315
#4912	*Sagittaria platyphylla*	USA: Tennessee, Lake	26 Oct 1983	Webb, D.H. #4912 (GH)	n/a	LC541635	n/a	LC541337
OS444968	*Sagittaria platyphylla*	USA: Ohio, Delaware	13 Aug 2011	Gardner, R.L. 7125 (OS444968)	LC541394	n/a	n/a	LC541338
BRK1936	*Sagittaria platyphylla*	USA: Alabama, Blount	23 Sep 2000	Keener, B.R. #1936 (UWAL0003459)	LC541427	LC541670	LC541726	LC541373
Chen	*Sagittaria potamogetifolia*	Garden-origin	n/a	n/a	JF781085	JF975438	JF781058	JF780972
Chen	*Sagittaria pygmaea*	China: Sichuan, Meishan	n/a	n/a	n/a	JF975439	JF781059	JF780973
TNS766316	*Sagittaria pygmaea*	Japan: Kyoto	10 Jul 2005	Tsugaru, T. et al. 1957 (TNS766316)	LC541395	LC541636	LC541691	LC541339
OS401844	*Sagittaria rigida*	USA: Ohio, Lucas	04 Aug 1998	Schneider, G. 1998:115 (OS401844)	LC541396	LC541637	LC541692	LC541340
OS412557	*Sagittaria rigida*	USA: Ohio, Lucas	09 Jul 2003	Gardner, R.L. 4538 (OS412557)	LC541397	LC541638	LC541693	LC541341
TUR327393	*Sagittaria sagittifolia*	Finland: Varsinais-Suomi	07 Aug 1991	Laine, U. & Rautiainen, P. s.n. (TUR327393)	LC541414	LC541657	LC541712	LC541359
SL31	*Sagittaria sprucei*	Peru: Loreto	07 Jul 2003	Lehtonen, S. & Rodriguez Arevalo 31 (TUR)	EF088104	n/a	n/a	EF088055
GH16693	*Sagittaria subulata*	USA: New York, Columbia	15 Aug 2001	Hellquist, C.B. 16693 & Brotzman, H. (GH)	LC541400	LC541641	LC541696	LC541344
Chen	*Sagittaria trifolia*	China: Sichuan, Chengdu	n/a	n/a	JF781083	JF975436	n/a[b]	JF780970

Table 1 (continued)

No	Taxon	Locality	Date	voucher	*matK*	*psbA*	*rbcL*	ITS
TD5636	*Sagittaria trifolia*	Garden-origin	06 Jul 2017	Tanaka, N. TD5636 (TNS)	LC541401	LC541642	LC541697	LC541345
YI1458	*Sagittaria trifolia*	Japan: Yamagata, Tainai	11 Sep 2010	Ito, Y. YI1458 (TNS)	LC541402	LC541643	LC541698	LC541346
YI2162	*Sagittaria trifolia*	Japan: Okayama, Akaiwa	03 Aug 2015	Ito, Y. YI2162 (TNS)	n/a	LC541644	LC541699	LC541347

[a] The specimen is deposited under an apparently wrongly identified name of *Sagittaria graminea*

[b] JF781056 was excluded due to its apparent contamination

related genera of *Sagittaria* (Chen et al. 2012), were used as outgroup taxa.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from silica gel-dried leaf tissue using the CTAB method as described in Ito et al. (2010). Three regions of ptDNA (*matK*, *psbA*, and *rbcL*) and nrITS were PCR amplified with the following primers: matK-390F (Cuénoud et al. 2002) and matK-1520R (Whitten et al. 2000) for *matK*; Psn3F and Psb3R (Chen et al. 2012) for *psbA*; rbcL-F1F (Wolf et al. 1994) and rbcL-1379R (Little and Barrington 2003) for *rbcL*; and ITS-4 and ITS-5 for nrITS (Baldwin 1992). PCR amplification was conducted using TaKaRa Ex Taq polymerase (TaKaRa Bio, Shiga, Japan). PCR cycling conditions were: initial denaturation at 94 °C for 60 s; followed by 30 cycles of 94 °C for 45 s, 52 °C for 30 s, 72 °C for 60 s, with a final extension of 72 °C for 5 min. PCR products were cleaned using ExoSAP-IT purification (GE Healthcare, Piscataway, New Jersey), and then amplified using Big Dye Terminator ver. 3.1 (Applied Biosystems, Foster City, California) using the same primers as those used for the PCR amplifications. DNA sequencing was performed with a 3130xl Genetic Analyzer (Applied Biosystems). Automatic base-calling was verified by eye in Genetyx-Win ver. 3 (Software Development Co., Tokyo, Japan). All sequences generated in the present study have been submitted to the DNA Data Bank of Japan (DDBJ), which is linked to GenBank. The accession numbers and voucher specimen information are presented in Table 1.

Molecular phylogenetic analysis

Sequences were aligned using Mafft ver. 7.058 (Katoh and Standley 2013) and then inspected manually. Indels were not coded because length variations were either ambiguous (nrITS) or observed only in *S. graminea* GH215 (a 9 bp-long insertion in *matK*). Accessions for ten *Sagittaria* spp. generated by Chen et al. (2012) were downloaded and included in our data sets except for *S. isoetiformis*, for which the ITS sequence (JF780976) apparently represents a contaminated sample or submission error, being identical to a sequence of *S. latifolia* (JF780975). Phylogenetic analyses were independently performed for ptDNA (*matK*, *psbA*, and *rbcL*) and nrITS data sets to identify possible incongruence between the different genomic regions. All 74 ingroup and the two outgroup accessions were included in the ptDNA data set, whereas the *Caldesia* outgroup sequence was excluded from the fast-evolving nrITS data set to allow more accurate alignment.

Phylogenies were reconstructed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI; Yang and Rannala 1997). In the MP analysis, conducted in PAUP* ver. 4.0b10 (Swofford 2002), a heuristic search was performed with 100 random addition replicates and tree-bisection-reconnection (TBR) branch swapping, with the MulTrees option in effect. The MaxTrees option was set at 100,000. Bootstrap analyses (Felsenstein 1985) were performed using 1,000 replicates with TBR branch swapping and simple addition sequences. The MaxTrees option was set at 1,000 to avoid entrapment in local optima. For the ML analysis, we used the RAxML BlackBox online server (Kozlov et al. 2019), which supports GTR-based models of nucleotide substitution (Stamatakis 2006). The maximum likelihood search option was used to find the best-scoring tree after bootstrapping. The gamma model of rate heterogeneity was applied as default. Statistical support for branches was calculated by rapid bootstrap analysis of 100 replicates (Stamatakis et al. 2008).

BI analyses were conducted with MrBayes ver. 3.2.2 (Ronquist and Huelsenbeck 2003; Ronquist et al. 2012) run on the CIPRES portal (Miller et al. 2010) after the best models had been determined in MrModeltest ver. 3.7 (Nylander 2002); these models were GTR + I + G and HKY + G for ptDNA and nrITS data sets, respectively. Analyses were run for 3,000,000 generations for ptDNA and nrITS data sets, respectively, sampling every 1,000 generations and discarding the first 25% as burn-in. The convergence and effective sampling sizes (ESS > 200 of all the parameters) were checked in Tracer ver. 1.6 (Rambaut et al. 2014). All trees were visualized using FigTree ver. 1.3.1 (Rambaut 2009). Nodes were recognized as strongly (≥ 90% MP bootstrap support (BS), ≥ 90% ML BS and ≥ 0.95 posterior probability (PP)), moderately (≥ 70% MP BS, ≥ 70% ML BS and ≥ 0.9 PP), or weakly (< 70% MP BS, < 70% ML BS and < 0.9 PP) supported. The data matrices and the MP, RAxML and BI trees were deposited in Treebase (TB2:S26217).

Molecular species delimitation

A Bayesian coalescent method of species delimitation was performed using STACEY (Jones 2017), which is an extension of DISSECT (Jones et al. 2015). Species tree estimation was performed in STACEY and species delimitation was conducted in SpeciesDelimitationAnalyser (Jones et al. 2015; Jones 2017). STACEY was implemented in BEAST ver. 2.4.4 (Bouckaert et al. 2014; Drummond and Rambaut 2007; Drummond et al. 2006). We ran STACEY using a multilocus data set (ptDNA and nrITS) with all ingroup species; outgroup species were excluded to avoid rate differences and hidden substitutions between ingroup and outgroup species (Bengt Oxelman, personal communication, November 22, 2016). We performed two independent runs of MCMC chains with ten million generations, sampling every 1,000 generations. Convergence of the stationary distribution was checked by visual inspection of plotted posterior estimates using Tracer ver. 1.6 (Rambaut et al. 2014). After discarding the first 1,000 trees as burn-in, the samples were summarized in the maximum clade credibility tree using TreeAnnotator ver. 1.6.1 (Drummond and Rambaut 2007) with a posterior probability limit of 0.5 and summarizing of mean node heights. The results were visualized using FigTree ver. 1.3.1 (Rambaut 2009).

Species tree and biogeographic analyses

Biogeographical reconstructions were performed using the species tree generated by BEAST. STACEY was run using a multilocus dataset (ptDNA and ITS) utilizing all 74 ingroup terminals and the outgroups assigned to 31 operational taxonomic units (OTUs), 29 of which were retrieved as species or minimal clusters in the STACEY analysis. The furthest outgroup was trimmed using RASP v.4.2 (Yu et al. 2020). Reconstruction of historical biogeography was performed using BioGeoBEARS (Matzke 2013) implemented in RASP v.4.2 (Yu et al. 2020). Model fit was assessed using the Akaike information criterion and likelihood-ratio tests. “Max areas” was set to the default (two). The following six biogeographic areas were defined: (A) Europe; (B) Africa; (C) temperate Asia; (D) tropical Asia; (E) North America; (F) South America (Fig. 1). Central America is included in North America because no species endemic to Central America are known and some North American species extend to Mexico and the West Indies, i.e., *S. demersa* J.G. Sm., *S. graminea*, and *S. longiloba* Engelm. ex J.G. Sm. (Haynes and Hellquist 2000). *Sagittaria longiloba*, previously known from North and Central America (Nicaragua) (Haynes and Hellquist 2000), was coded to be distributed in North and South America because one of our samples (SL466) was collected from Venezuela (Fig. 1, Table 1).

Results

Molecular phylogeny

The ptDNA data set comprising three genes included 3,107 aligned characters, of which 201 were parsimony-informative. Analysis of this data set overflowed the imposed limit of 100 000 MP trees (tree length = 511 steps; consistency index = 0.77; retention index = 0.90). The strict consensus of these trees, the RAxML tree, and the MrBayes 50% majority rule consensus tree showed no strongly supported incongruences; thus, only the Bayesian tree is presented here (Fig. 2a). The nrITS data set included 751 aligned characters, of which 259 were parsimony-informative. Analysis of this data set overflowed the imposed limit of 100,000 MP trees (tree length = 668 steps; consistency index = 0.71; retention index = 0.91). The strict-consensus of these trees, the ML tree and the Bayesian 50% majority rule consensus tree showed no incongruent phylogenetic relationships; thus, only the MrBayes tree is presented here (Fig. 2b).

The monophyly of the genus *Sagittaria* was highly supported (98% MP BS, 93% ML BS, 1.0 PP in Fig. 2a). While *Sagittaria guayanensis* (clade 1) was weakly resolved as sister to the rest of the genus in ptDNA with *S. planitiana* G. Agostini (clade 2) resolved as the sister to the rest of the species, the two species were clustered in nrITS (84% MP BS, 100% ML BS, 1.0 PP) and resolved together as sister to all other *Sagittaria*. Clade 5 plus *S. montevidensis* SL180 and *S. sprucei* Micheli were resolved as sister to clades 6–22 in both ptDNA and nrITS (98% MP BS, 100%ML BS; 1.0 PP in Fig. 2a; 99% MP BS, 100% ML BS; 1.0 PP in Fig. 2b). *Sagittaria longiloba* was weakly clustered with clades 6–14 in ptDNA (0.97 PP in Fig. 2a) but resolved as sister to clades 16–22 in nrITS (87% MP BS, 91% ML BS, 1.0PP in Fig. 2b). While clades 6–9 were weakly supported as monophyletic in ptDNA (56% MP BS, 70% ML BS, 1.0 PP in Fig. 2a), clades 7–9 were resolved with clades 10–14 in nrITS (100% MP BS, 96% ML BS, 1.0PP in Fig. 2b). Clades 16–18 formed a monophyletic group in both analyses (100% MP BS, 100% ML BS, 1.0 PP in both Fig. 2a, b) and were resolved as sister to clades 19–22 (76% MP BS, 81% ML BS, 1.0 PP in Fig. 3a; 100% MP BS, 97% ML BS, 1.0 PP). *Sagittaria sagittifolia* L. was weakly clustered with clade 19 in ptDNA (< 50% MP BS, < 70%ML BS; 1.0 PP in

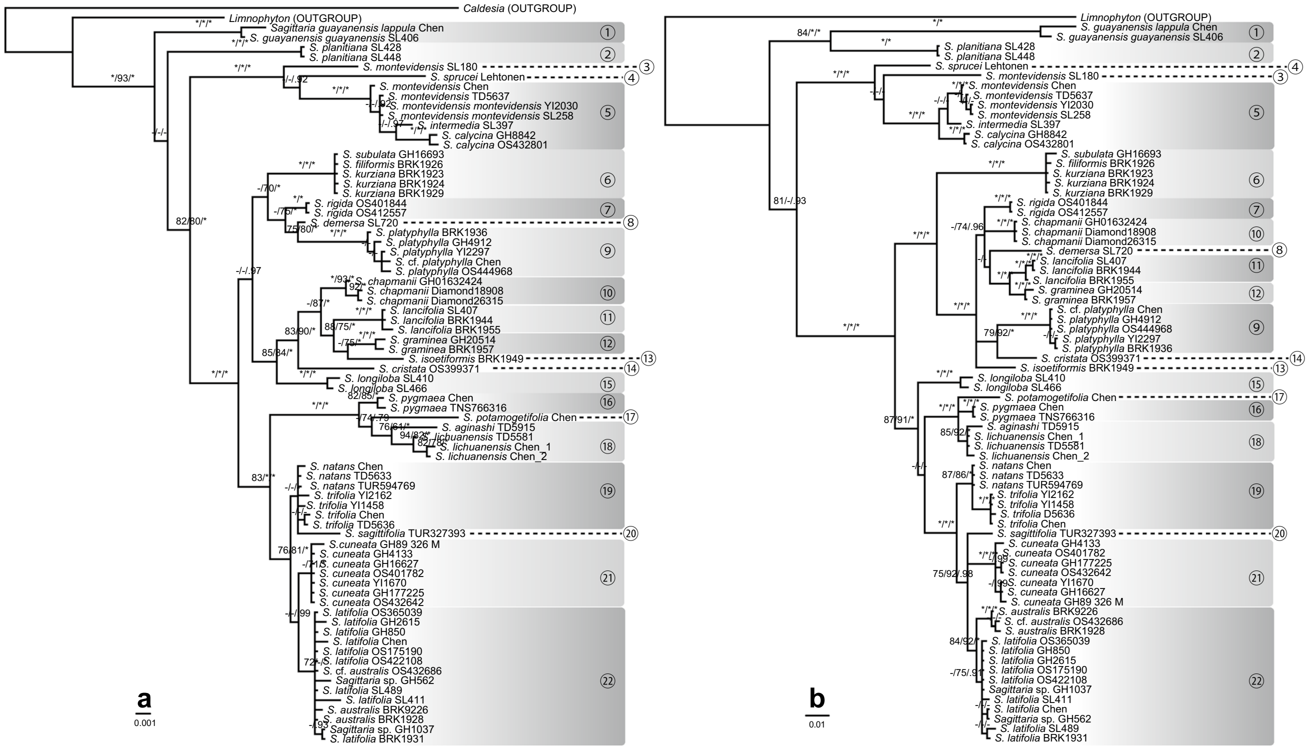

Fig. 2 MrBayes trees of *Sagittaria* based on **a** ptDNA and **b** nrITS datasets. Samples collected in this study are associated with the specified vouchers. Branch lengths are proportional to the number of substitutions per site as measured by the scale bar. Values above the branches represent the maximum parsimony and maximum likelihood bootstrap support values (MP BS/ML BS), and Bayesian posterior probabilities (PP). BS < 70% and PP < 0.9 are indicated by hyphens while those of ≥ 90% and ≥ 0.95 are shown as asterisks. Well-supported clades are highlighted by gray rectangles with numbers

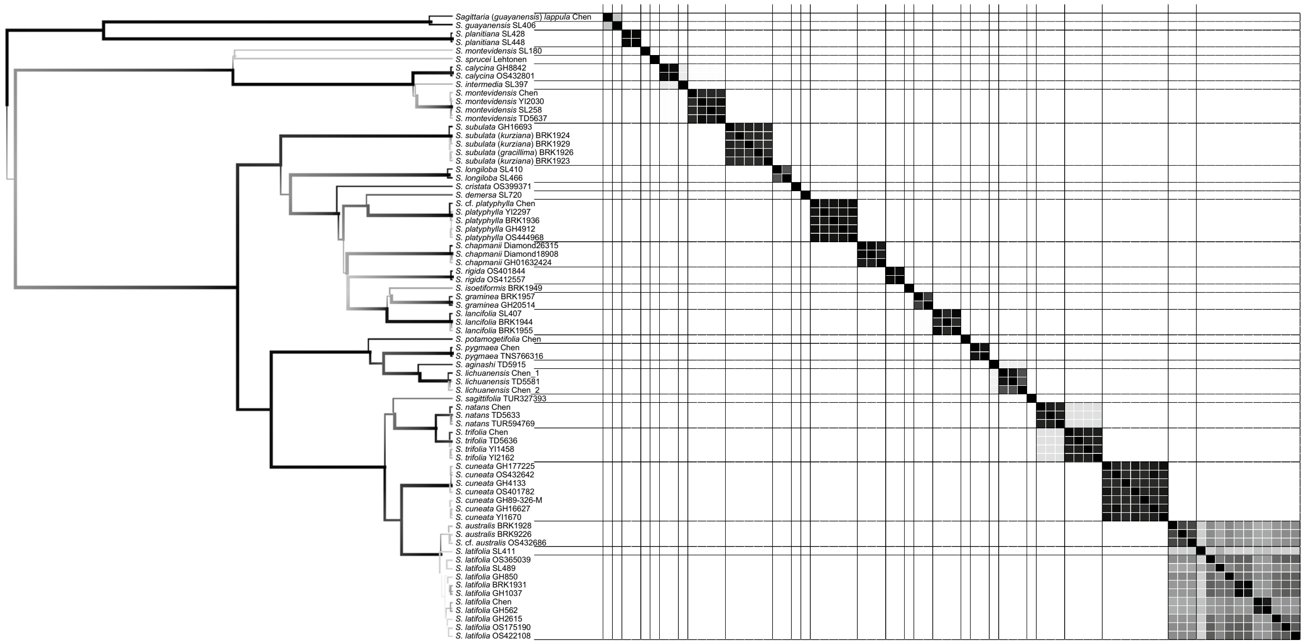

Fig. 3 Maximum sum of clade credibility SMC-tree based on multi-locus (ptDNA and nrITS) data set of *Sagittaria* from BEAST 2 analysis and similarity matrix from STACEY analysis. Posterior probabilities from BEAST 2 are given for major clades on the branches. The squares in the matrix represent posterior probabilities (white = 0, black = 1) for pairs of individuals belonged to the same cluster. The horizontal lines represent species boundaries suggested by STACEY analysis. Taxa that receive taxonomic changes are shown with parentheses (see "Discussion" for more detail)

Fig. 2a) but was clustered with clades 21–22 in nrITS (75% MP BS, 92% ML BS, 0.98 PP in Fig. 2b).

Species delimitation using STACEY

SpeciesDelimitationAnalyser generated 1,527 patterns of clusters from the MCMC runs. Classifications with 28 or fewer *Sagittaria* minimal clusters (species) received higher posterior probabilities (Table S1). Under these classifications, the species delimitation among *S. latifolia* and *S. australis* as well as the status of the two subspecies of *S. guayanensis* remained unclear. The similarity scores among these clusters seem to be low enough to support distinct species, i.e., the similarity score of *S. australis* vs. *S. latifolia* SL411 was 0.26–0.27; *S. latifolia* SL411 vs. *S. latifolia* excluding SL411 was 0.24–0.42; *S. australis* vs. *S. latifolia* excluding SL411 was 0.43–0.60; *S. guayanensis* subsp. *guayanensis* vs. *S. guayanensis* subsp. *lappula* was 0.31 (Table S2). Therefore, for further analyses we recognized the two subspecies of *S. guayanensis*, *S. australis*, *S. latifolia* and *S. latifolia* SL411 as species. Hence, a total of 29 species with the posterior probability of 0.10 PP was accepted (Fig. 3).

Biogeography

The BioGeoBEARS tool in RASP assigned the DEC + j scenario as the best model (AICc_wt = 0.52), followed by DIVALIKE + J (AICc_wt = 0.35) and BAYAREALIKE + J (AICc_wt = 0.13). Under the DEC + j scenario, the ancestral area of the root of *Sagittaria* (node I) was resolved as South America (0.65 PP) (node I in Figs. 4, S1). The ancestral area of node II was South America (0.71 PP) (Figs. 4, S1). The ancestral area of node III was North America (0.55 PP) (Figs. 4, S1). The ancestral area for the clade containing Asian species and *S. latifolia* (node IV) was North America (0.59 PP). The ancestral area for Asian species (node V) was tropical Asia (0.54 PP), or temperate Asia (0.34 PP). The ancestral area for *S. calycina*, *S. intermedia*, and *S. montevidensis* (node VI) was South America (0.80 PP). The ancestral area for the two subspecies of *S. guayanensis* (node VII) was North and South America (0.61 PP).

Discussion

Phylogeny of *Sagittaria*

Molecular phylogenetics of *Sagittaria* have been previously studied by Keener (2005), who sampled the 5S-NTS marker for 73 accessions representing 38 taxa, and Chen et al. (2012) in their phylogenetic study of Alismataceae by using nrITS and ptDNA (*matK*, *psbA*, and *rbcL*) with a set of 10 *Sagittaria* species. Here, we gathered 74 *Sagittaria* samples, including eight samples from Keener (2005), three from Lehtonen and Myllys (2008), and nine from Chen et al. (2012), and conducted the most comprehensive molecular phylogenetic analysis of the genus so far. The results showed no significant topological incongruence between the previous results, i.e., (i) *S. guayanensis* and *S. montevidensis* were among the early diverged species (clades 1–5 in Fig. 2; Chen et al. 2012; Keener 2005); (ii) a clade containing *S. potamogetifolia* Merr. and *S. pygmaea* Miq. was recovered (clades 16–18 in Fig. 2; Chen et al. 2012; Keener 2005); (iii) a clade containing *S. latifolia*, *S. natans*, and *S. trifolia* was recovered (clades 19–22 in Fig. 2; Chen et al. 2012; Keener 2005, Fig. 1a, b except *S. isoetiformis* for which the ITS sequence apparently represents an error, being identical to a sequence of *S. latifolia*).

Cryptic diversity in *Sagittaria*

Sagittaria montevidensis is currently considered as a widely distributed species ranging from North America to Mexico and South America (Haynes and Hellquist 2000). Keener (2005) divided this species of four subspecies into two species, each having two subspecies, i.e., *S. calycina* in North and Central America, and *S. montevidensis* in South America. *Sagittaria intermedia* from Central America is also clustered with these two species in our molecular phylogeny. Given the morphological similarity, *S. sprucei* from South America was suggested to be related to these species (Keener 2005) and our phylogeny confirmed this.

The South American samples identified as *S. montevidensis* were split so that the Bolivian sample (SL180) was resolved highly distinct from the others (YI2030 from Argentina, SL258 from Paraguay, and two garden-origin). Two varieties of *S. montevidensis* are generally recognized: var. *chilensis* from temperate South America and var. *montevidensis* from tropical South America (Bogin 1955; Keener 2005). They can be distinguished by the absence or smaller size of the basal lobes, and by the carpellate pedicels being inflated at the base of the flowers in var. *chilensis*, whereas in var. *montevidensis* the basal lobes are longer than or equal to the remainder of the blade, and pedicels of carpellate flowers are merely thickened at the base of the flowers. The sample from Bolivia had large lobes and merely thickened pedicels and therefore cannot represent var. *chilensis*. In addition, it lacked the purple spots at the base of the petals, a character typical for *S. montevidensis*, but instead had yellow spots. However, the current sampling and limited herbarium material available to us does not allow well justified taxonomic conclusions at the moment, but further taxonomic studies in the South American *S. montevidensis* are clearly needed to clarify the species delimitation within this apparent species complex.

Fig. 4 Ancestral range reconstruction on the phylogenetic tree of *Sagittaria* with the best fitting DEC+j model in BioGeoBEARS. The branch width and color indicate posterior priorities (thick black lines with higher probabilities). The areas inferred with the highest posterior probabilities are shown; those which have similar posterior probabilities for different areas inferred are shown below. The dotted arrows indicate colonization events out of South America. The closed squares on the right indicate distribution areas for each species. Area codes are as follows: **a** Europe, **b** Africa, **c** Temperate Asia, **d** Tropical Asia, **e** North America, **f** South America. The roman numerals at nodes are mentioned in the text. The outgroup was trimmed

Sagittaria latifolia is currently treated as containing two varieties (var. *latifolia* and var. *pubescens*) distinguished by the presence or absence of pubescence over the plant (Bogin 1955). While Ecuadorian specimens were identified as *S. latifolia* var. *latifolia* (Holm-Nielsen and Haynes 1986), recent floras of North America, i.e., Crow and Hellquist (2000), Haynes and Hellquist (2000), and Keener (2005), tend not to recognize the varieties, adding that “many (specimens) from the southeastern United States are pubescent” (Haynes and Hellquist 2000). We found no significant genetic differences among the North and South American samples, whereas the species delimitation analysis distinguished—although weakly—the Mexican sample (SL411) from the other North and South American samples (Fig. 3). Sculthorpe (1967) pointed out the presence of sporadic populations of *S. latifolia* in Central America which are discontinuous from the main distribution area in North America, indicating geographic isolation in Mexican populations including our sample. The broad and somewhat discontinuous distribution of the *S. latifolia* complex is a subject for future taxonomic studies.

Evidence for colonization out of South America

Chen et al. (2012) resolved the paleotropical clade composed of *Astonia* S.W.L. Jacobs, *Limnophyton*, and *Wiesneria* Micheli as the sister to *Sagittaria* and inferred Africa and North America as ancestral areas for *Sagittaria*. This contrasts with our result of a South American origin for the genus. Unlike Chen et al. (2012), we included only one outgroup, *Limnophyton*, in our biogeographic analysis and the results may have been different if a broader taxonomic sampling had been used. However, the South American *Sagittaria* are much better sampled in our study compared to Chen et al. (2012), and their phylogenetic position clearly indicates that the deepest split in the genus occurred in South America. It therefore seems reasonable to assume that the diversification of modern *Sagittaria* began in South America, even if the ancestor had reached South America by dispersal from Africa.

Our analysis revealed multiple events of colonization out of South America, leading to (i) the first invasion to North America (node III in Fig. 4); (ii) the second invasion to North America at the most recent common ancestor for *S. calycina* and its allies (although the clade of *S. calycina* and *S. intermedia* is less-supported); and (iii) a range expansion of the most recent common ancestor for *S. guayanensis* and its allies to North America and a subsequent intercontinental dispersal of *S.* (*guayanensis* subsp.) *lappula* to paleotropics (Fig. 4). Subsequent speciation occurred in North America, where at least ten species evolved (Fig. 4). Furthermore, we infer the range expansion from North America to East Asia at node V and another dispersal event from North America to Europe with a subsequent range expansion to Asia, resulting in *S. sagittifolia*, *S. natans* and *S. trifolia* (Fig. 4).

Some *Sagittaria* species are widely distributed in the Americas, such as *S. lancifolia*, *S. latifolia*, and *S. longiloba* (Haynes and Hellquist 2000; Holm-Nielsen and Haynes 1986). Our biogeographic analysis showed North America for the most recent common ancestors for these species and their sister species (Fig. 4), suggesting that, at species level, range expansions from North America back to South America occurred at least three times.

Implication for taxonomy and evolution of *Sagittaria* species

Sagittaria guayanensis is widespread in most tropical countries of the world and is divided into two subspecies: *S. guayanensis* subsp. *guayanensis* in Neotropics (Western hemisphere) and *S. guayanensis* subsp. *lappula* in Paleotropics (Eastern hemisphere) (Haynes and Hellquist 2000; Holm-Nielsen and Haynes 1986). The monophyly of *S. guayanensis* was confirmed by Keener (2005) as well as our ptDNA and nrITS phylogenies (Fig. 2). However, our species delimitation analysis suggested that the subspecies could be recognized as two different species (Fig. 3). We therefore recommend restoring species status of *S. guayanensis* subsp. *lappula* as S. *lappula* D. Don.

Sagittaria filiformis, *S. kurziana*, and *S. subulata* are morphologically highly similar, narrowly distributed endemics to southeastern North America (Haynes and Hellquist 2000). Keener (2005) as well as we found no, or very limited genetic variation among these taxa (Fig. 2). We therefore propose treating these taxa as varieties of *S. subulata*: *Sagittaria subulata* var. *gracillima* (S. Watson) J.G. Sm., *Sagittaria subulata* var. *kurziana* (Glück) Bogin, and *Sagittaria subulata* var. *subulata*, respectively.

Conclusions

We provided new phylogenetic insights into the diversity and biogeography of *Sagittaria*, a nearly cosmopolitan genus with greatest diversity in the Americas. Our molecular phylogenetic analyses based on ptDNA and nrITS data sets using an expanded taxon sampling revealed cryptic diversity in widely distributed *S. latifolia* and *S. montevidensis*. A previously inferred African–North American origin of the genus was revised to an ancestral distribution in South America, with deepest diversifications occurring there subsequently followed by speciation in North America. Three colonization events occurred out of South America, towards North America and Asia. At species level, *S. lancifolia*, *S. latifolia*, and *S. longiloba* represent range expansions back to South America. Eurasia has been occupied twice, possibly by means of relatively short-distance circumpolar dispersal from North America. The biogeographic insights revealed in this study support the importance of inter-continental dispersal in widely distributed aquatic plants. Our study demonstrates the importance of broad sampling of widespread species for addressing cryptic diversity in aquatic plants.

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