DISENTANGLING RETICULATE EVOLUTION OF NORTH TEMPERATE HAPLOSTEMONOUS *LUDWIGIA* (ONAGRACEAE)[1,2]

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ABSTRACT

While it is known that whole genome duplication (WGD) and reticulate evolution play important roles in plant evolution, the origins and evolutionary histories of most polyploid and reticulate groups are still poorly known. The North Temperate haplostemonous (NTH) *Ludwigia* L. (sections *Isnardia* (L.) W. L. Wagner & Hoch, *Ludwigia*, *Microcarpium* Munz, and *Miquelia* P. H. Raven) group, characterized by having 4-merous and haplostemonous flowers, pluriseriate and free seeds, glabrous and convex nectaries, and a north-temperate distribution, is a polyploid complex (2×, 4×, 6×, and 8×) of 24 species with frequent reports of inter- and intrasectional hybridization. Although earlier biosystematics studies postulated some evolutionary scenarios and recent molecular phylogenetic studies have partially tested these propositions, the full history of their reticulate evolution remains puzzling. In this study, we sequenced four chloroplast regions (*rpL16*, *rpoB-trnC*, *trnL-trnF*, and *ycf6-psbM*) and conducted extensive molecular cloning of the biparentally inherited single-copy nuclear *PgiC* gene (376 clones in total), sampling 23 of the 24 NTH *Ludwigia* species whose chromosome numbers and ploidy levels were confirmed. Both the chloroplast and *PgiC* trees include strongly supported sister clades of section *Ludwigia* (four diploid species) and the "*Microcarpium* complex" (composed of sections *Isnardia*, *Microcarpium*, and *Miquelia*), which together are sister to the rest of *Ludwigia*. In the *PgiC* tree, eight clades are identified within the *Microcarpium* complex, with four clades including no extant diploid species. Neither sections *Isnardia* nor *Microcarpium* are monophyletic, while the monospecific section *Miquelia* has a hybrid origin. By integrating our phylogenetic trees with previous cytological hypotheses, the reticulate evolution of NTH *Ludwigia* is disentangled and four to eight extinct diploid species are inferred. Ancestral area reconstruction supports a North American origin of *L. ovalis* whose current East Asian distribution reflects a relict of the Arcto-Tertiary Geoflora. Based on our results, we propose to synonymize sections *Microcarpium* and *Miquelia* under the expanded section *Isnardia*.

Key words: Aquatic plants, chloroplast phylogeny, chromosome cytology, glucose-6-phosphate isomerase cytosolic (*PgiC*) gene, hybridization, *Ludwigia*, Onagraceae, polyploids.

Whole genome duplication (WGD) and reticulate evolution are both common and evolutionarily important in the angiosperms (Stebbins, 1971; Arnold, 1997; Levin, 2002; Soltis et al., 2014). The complexity of polyploid taxa and their reticulate evolution has impeded efforts to elucidate the origins and evolutionary relationships of these taxa (Rieseberg & Willis, 2007; Soltis et al., 2014; Barker et al., 2016). Earlier cytological studies contributed greatly to the understanding of WGD and reticulate evolution in some plant lineages (e.g., Wagner, 1954; Peng, 1983, 1990; Ramamoorthy & Zardini, 1987). Molecular phylogenetic analyses have also provided insights into the origins and relationships of some complicated taxa (e.g., Ford & Gottlieb, 2007; Jiao & Paterson, 2014). Several recent studies have shown that combining both molecular and cytological analyses provides even better resolution to untangle the evolutionary puzzle in polyploid and/or reticulate groups (Lipman et al., 2013; Mandák et al., 2018).

Ludwigia L., the sole genus in Onagraceae subfamily Ludwigioideae, is an intensively studied polyploid complex comprising 83 predominately wetland species

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[2] C.-I. P., K.-F. C., and P. C. H. conceived the study; C.-I. P. and K.-F. C. collected the species from the United States and Taiwan; C.-I. P. took color photographs of the study plants from the wild and the Academia Sinica greenhouse; Y. K. examined the somatic chromosomes; H. A. Y. extracted DNA, amplified the chloroplast regions, and cloned the *PgiC* region; K.-F. C. and J. C. B. contributed analysis tools; S. H. L. performed phylogenetic analyses and trait evolution analyses; S. H. L. prepared figures and tables, and prepared and archived the sequence data to National Center for Biotechnology Information (NCBI); S. H. L. and K.-F. C. wrote the manuscript; all authors read and approved the final manuscript.

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with a worldwide distribution (Munz, 1942; Raven, 1963; Eyde, 1977; Raven & Tai, 1979; Peng, 1983; Wagner et al., 2007; Pesamosca & Boldrini, 2015; Liu et al., 2017). The North Temperate haplostemonous (NTH) *Ludwigia* group, characterized by 4-merous and haplostemonous flowers and north-temperate distribution, includes 24 species that have been classified in section *Ludwigia*, section *Isnardia* (L.) W. L. Wagner & Hoch, section *Microcarpium* Munz, and section *Miquelia* P. H. Raven (Table 1; Raven, 1963; Wagner et al., 2007). The NTH *Ludwigia* group is relatively well studied, including analyses on its floral, fruit, and seed anatomy, palynology, embryology, reproductive biology, and cytology (Eyde, 1977, 1978, 1981; Raven, 1979; Raven & Tai, 1979; Praglowski et al., 1983; Peng & Tobe, 1987; Peng, 1988, 1989; Tobe et al., 1988; Peng et al., 2005).

Ludwigia sect. *Ludwigia* (Fig. 1A) includes four diploid species ($2n = 2\times = 16$), distinguished from other NTH *Ludwigia* by the combination of alternate leaves, tuberous roots, relatively larger petals, apically dehiscent capsules, and pollen release in tetrads or polyads (Raven & Tai, 1979; Peng, 1989; Wagner et al., 2007). The other three sections constitute the *Microcarpium* complex (Eyde, 1981), comprising five diploids, 10 tetraploids ($2n = 4\times = 32$), four hexaploids ($2n = 6\times = 48$), and one octoploid ($2n = 8\times = 64$) (Raven & Tai, 1979; Peng, 1988; Peng et al., 2005). *Ludwigia ovalis* Miq. (Fig. 1B) of the monospecific section *Miquelia* is a tetraploid native to East Asia, characterized by alternative leaves, apetalous flowers, irregularly dehiscent capsules, pollen shed in monads, and enlarged seed raphe (Wagner et al., 2007). *Ludwigia ovalis* is the only NTH *Ludwigia* not native to America.

Section *Isnardia* (five species; Fig. 1C–E), previously known as section *Dantia* Munz, is characterized by having fibrous roots, opposite leaves, apetalous or small-petaled flowers, irregularly dehiscent capsules, and pollen shed in monads or tetrads (Schmidt, 1967; Peng et al., 2005; Wagner et al., 2007). Peng et al. (2005) extensively examined the morphology, geographical distribution, breeding system, and chromosome configuration of section *Isnardia*, including also cytology and pollen stainability of artificial interspecific hybrids. They reported that the genomes in the section can be identified based on the ability of the chromosomes in the genome to pair as bivalents with chromosomes from other genomes at meiotic metaphase I, shown in their artificial interspecific hybrid experiments. Peng et al. (2005) then identified and hypothesized that four genomes, designated A, B, C, and D (Schmidt, 1967), have been involved in the formation of section *Isnardia*. The genome of the widely distributed diploid species *Ludwigia palustris* (L.) Elliott was designated as AA. This species was hypothesized to contribute the A genome to the tetraploid *L. spathulata* Torr. & A. Gray (AADD), following hybridization. The source of the D genome in *L. spathulata* was not known, however, since there was no extant diploid DD species. Both *L. arcuata* Walter (BBCC; 4×) and *L. palustris* were hypothesized to contribute to the origins of the hexaploids *L. repens* J. R. Forst. (AABBCC) and *L. brevipes* (Long) Eames (AABBCC). Despite both having the same genomic complement, these two hexaploid species differ in many morphological traits, possibly resulting from independent and/or reciprocal hybridization events (Peng et al., 2005).

Section *Microcarpium* (14 species; Fig. 1F–I) differs from the other NTH *Ludwigia* sections by the combination of fibrous roots, alternate leaves, vestigial petalous or apetalous flowers, regularly or irregularly or apically dehiscent capsules, and pollen release in monads or tetrads (Raven & Tai, 1979; Peng, 1989; Wagner et al., 2007). In their review of the cytology of *Ludwigia*, Raven and Tai (1979) first proposed hypotheses about the origins of polyploids in this section. Additional systematic, cytological, and experimental hybridization analyses allowed Peng (1988, 1989) to refine the work of Raven and Tai (1979). To avoid confusion with genomes A, B, C, and D already assigned to section *Isnardia* (Schmidt, 1967), Peng (1988) assigned the F genome to both *L. linearis* Walter (FF; 2×) and *L. linifolia* Poir. (FF; 2×) to reflect their morphological and distributional similarity, as well as high seed set, high pollen stainability, and the formation of six to eight bivalents in meiotic metaphase in experimental hybrids (Fig. 2B). Peng (1988) also hypothesized that *L. curtissii* Chapm. (FFGGHHII; 8×) inherited the F genome from *L. linifolia* or *L. linearis*, and obtained the G, H, and I genomes from *L. simpsonii* Chapm. (GGHHII; 6×). The diploid *L. microcarpa* Michx. (GG; 2×) and unidentified/extinct species with HHII likely contributed genomes to *L. simpsonii* (Peng, 1988), while the opposite or subopposite leaves near stem bases and a somewhat ascending to prostrate habit in *L. simpsonii* suggest its affinity with a possibly extinct member of section *Isnardia* (Peng, 1989). The seven tetraploid species of section *Microcarpium* (*L. glandulosa* Walter, *L. lanceolata* Elliott, *L. pilosa* Walter, *L. polycarpa* Short & R. Peter, *L. ravenii* C.-I Peng, *L. sphaerocarpa* Elliott, and *L. suffruticosa* Walter) are closely related and form what appears to be a homogamic complex (Peng, 1988), but the genomic origins of these tetraploids remain unclear. Here, we assign unidentified genome as "X" for the latter discussion (i.e., the seven tetraploid species of section *Microcarpium* are XXXX). Based on its irregular seed surface pattern, Peng (1988) also hypothesized that hybridization between the interfertile tetraploids was responsible for the origin of the highly polymorphic *L. sphaerocarpa* (Fig. 2B). The hexaploid *L. alata* Elliott (GGXXXX)

Table 1. Taxon sampling, voucher information (collector[s], collection number, and location), sample number, ploidy level, and GenBank accession numbers. Voucher specimens are deposited in the Herbarium of the Biodiversity Research Center, Academia Sinica (HAST). The information of ploidy levels was extracted from Raven and Tai (1979), Peng (1988), and Peng et al. (2005). Asterisks indicate taxa for which ploidy level was confirmed by new chromosome counts (see Fig. 3).

Section (section code) / Taxon	Collector(s) and collection number	Location	Sample number(s)	Ploidy level	GenBank accession numbers				
					RPL16	*RPOB-TRNC*	*TRNL-TRNF*	*YCF6-PSBM*	*PGIC*
Section *Isnardia* (L.) W. L. Wagner & Hoch (ISN)									
L. arcuata Walter	*J. R. Abbott 22396*	U.S.A., Florida, Lafayette Co.	25	4*x*	MH177326	MH177370	MH198380	MH177414	MK820759-MK820778
L. brevipes (Long) Eames	*C.-I Peng 19434*	U.S.A., North Carolina, Cumberland, Rhodes Pond	58	6*x**	MH177327	MH177371	MH198381	MH177415	MK820779-MK820791
	C.-I Peng 19435	U.S.A., North Carolina, Cumberland	59	6*x*					MK820792-MK820802
L. palustris (L.) Elliott	*C.-I Peng 21008*	Taiwan, Academia Sinica	56	2*x*	MH177350	MH177394	MH198404	MH177438	MK820889
	K.-F. Chung 1901	U.S.A., Florida, Hendry Co.	22	2*x*	MH177349	MH177393	MH198403	MH177437	MK820888
L. repens J. R. Forst.	*C.-I Peng 18499*	U.S.A., Florida, Hillsborough Co.	55	6*x*	MH177355	MH177399	MH198409	MH177443	MK847327-MK847342
	C.-I Peng 18502	U.S.A., Florida, Dixie Co.	46	6*x*	MH177356	MH177400	MH198410	MH177444	MK847316-MK847326
	K.-F. Chung 1784	U.S.A., Florida, Collier Co.	23	6*x**	MH177354	MH177398	MH198408	MH177442	MK847278-MK847291
	K.-F. Chung 1795	U.S.A., Florida, Charlotte Co.	30	6*x*					MK847292-MK847303
	K.-F. Chung 1902	U.S.A., Florida, Hendry Co.	31	6*x*					MK847304-MK847315
L. spathulata Torr. & A. Gray	*C.-I Peng 19287*	U.S.A., Alabama	28	4*x*	MH177359	MH177403	MH198413	MH177447	MK847381-MK847388
Section *Microcarpium* Munz (MIC)									
L. alata Elliott	*T.-W. Hsu 11327*	U.S.A., Florida	10, 47	6*x**	MH177322	MH177366	MH198376	MH177410	MK820731-MK820736, MK820741-MK820748
	T.-W. Hsu 11365	U.S.A., South Carolina, Charleston Co.	11, 48	6*x**	MH177323	MH177367	MH198377	MH177411	MK820737-MK820740, MK820749-MK820756
L. curtissii Chapm.	*K.-F. Chung 1785*	U.S.A., Florida, Kirby S. Storter Park	32	8*x**	MH177328	MH177372	MH198382	MH177416	MK820815-MK820824
	K.-F. Chung 1895	U.S.A., Florida, Lee Co.	33	8*x*	MH177329	MH177373	MH198383	MH177417	MK820803-MK820814, MK820825-MK820831
	K.-F. Chung 1900	U.S.A., Florida, Hendry Co.	35	8*x**	MH177330	MH177374	MH198384	MH177418	MK820832-MK820841
L. glandulosa subsp. *brachycarpa* C.-I Peng	*O'Kennon 21312*	U.S.A., Texas, Wise Co.	57	4*x**	MH177331	MH177375	MH198385	MH177419	MK820842-MK820848
L. glandulosa Walter subsp. *glandulosa*	*J. R. Abbott 23537*	U.S.A., Florida, Liberty Co.	4	4*x**	MH177332	MH177376	MH198386	MH177420	MK820849-MK820854
L. lanceolata Elliott	*Anderson 24701*	U.S.A., Florida, Franklin Co.	52	4*x**	MH177335	MH177379	MH198389	MH177423	MK820856-MK820863
	Anderson s.n.	U.S.A., Florida	73	4*x**	MH177334	MH177378	MH198388	MH177422	MK820864-MK820868
L. linearis Walter	*J. R. Abbott 23369*	U.S.A., Florida, Levy Co.	2	2*x**	MH177336	MH177380	MH198390	MH177424	MK820869
	J. R. Abbott 23563	U.S.A., Florida, Bay Co.	3	2*x**	MH177337	MH177381	MH198391	MH177425	MK820870
L. linifolia Poir.	*J. R. Abbott 23623*	U.S.A., Florida, Escambia Co.	1	2*x**	MH177338	MH177382	MH198392	MH177426	MK820871

Table 1. Continued.

Section (section code) / Taxon	Collector(s) and collection number	Location	Sample number(s)	Ploidy level	GenBank accession numbers				
					RPL16	*RPOB-TRNC*	*TRNL-TRNF*	*YCF6-PSBM*	*PGIC*
L. microcarpa Michx.	*J. R. Abbott 17967*	U.S.A., Florida, Levy Co.	14	2*x**	MH177342	MH177386	MH198396	MH177430	MK820875
	K.-F. Chung 1783	U.S.A., Florida, Collier Co.	37	2*x*	MH177343	MH177387	MH198397	MH177431	MK820876
	K.-F. Chung 1786	U.S.A., Florida, Kirby S. Storter Park	38	2*x*	MH177344	MH177388	MH198398	MH177432	MK820877
	K.-F. Chung 1792	U.S.A., Florida, Collier Co.	44	2*x*	MH177345	MH177389	MH198399	MH177433	MK820878
	K.-F. Chung 1796	U.S.A., Florida, Charlotte Co.	39	2*x**	MH177346	MH177390	MH198400	MH177434	MK820879
	K.-F. Chung 1899	U.S.A., Florida, Lee Co.	40	2*x*	MH177347	MH177391	MH198401	MH177435	MK820880
L. pilosa Walter	*J. R. Abbott 23584*	U.S.A., Florida, Gulf Co.	49	4*x**	MH177351	MH177395	MH198405	MH177439	MK820890-MK820907
L. polycarpa Short & R. Peter	*Thompson s.n.*	U.S.A., Iowa, Story Co.	62	4*x**	MH177352	MH177396	MH198406	MH177440	MK847266-MK847271
L. ravenii C.-I Peng	*Wilbur & Ho 85172*	U.S.A., North Carolina, Catfish Lake	50	4*x**	MH177353	MH177397	MH198407	MH177441	MK847272-MK847277
L. simpsonii Chapm.	*K.-F. Chung 1790*	U.S.A., Florida, Collier Co.	42	6*x**	MH177357	MH177401	MH198411	MH177445	MK847343-MK847357, MK847370-MK847375
	K.-F. Chung 1794	U.S.A., Florida, Collier Co.	43	6*x**	MH177358	MH177402	MH198412	MH177446	MK847358-MK847369, MK847376-MK847380
L. sphaerocarpa Elliott	*J. R. Abbott 23370*	U.S.A., Florida, Levy Co.	12, 61	4*x**	MH177361	MH177405	MH198415	MH177449	MK847389-MK847416
	Anderson s.n.	U.S.A., Florida, Wakulla Co.	81	4*x**	MH177360	MH177404	MH198414	MH177448	MK847417-MK847434
L. suffruticosa Walter	*J. R. Abbott 23349*	U.S.A., Florida, Bradford Co.	51	4*x**	MH177362	MH177406	MH198416	MH177450	MK847441-MK847448
	K.-F. Chung 1907	U.S.A., Florida, Glades Co.	29	4*x*	MH177363	MH177407	MH198417	MH177451	MK847435-MK847440
Section *Ludwigia* L. (LUD)									
L. alternifolia L.	*J. R. Abbott 23613*	U.S.A., Florida, Escambia Co.	19	2*x**	MH177324	MH177368	MH198378	MH177412	MK820757
	Les 808	U.S.A., Connecticut	20	2*x*	MH177325	MH177369	MH198379	MH177413	MK820758
L. hirtella Raf.	*J. R. Abbott 23616*	U.S.A., Florida, Escambia Co.	27	2*x**	MH177333	MH177377	MH198387	MH177421	MK820855
L. maritima R. M. Harper	*J. R. Abbott 23333*	U.S.A., Florida, Bradford Co.	16	2*x**	MH177339	MH177383	MH198393	MH177427	MK820872
	J. R. Abbott 23565	U.S.A., Florida, Gulf Co.	17	2*x**	MH177340	MH177384	MH198394	MH177428	MK820873
	J. R. Abbott 23576	U.S.A., Florida, Bay Co.	18	2*x**	MH177341	MH177385	MH198395	MH177429	MK820874
L. virgata Michx.	*J. R. Abbott 23603*	U.S.A., Florida, Libery Co.	15, 71	2*x**	MH177364	MH177408	MH198418	MH177452	MK847449-MK847450
Section *Miquelia* P. H. Raven (MIQ)									
L. ovalis Miq.	*C.-I Peng 13859*	Taiwan, Fushan Botanical Garden	72	4*x*	MH177348	MH177392	MH198402	MH177436	MK820881-MK820887
Outgroups									
L. adscendens (L.) H. Hara	*C.-I Peng 21007*	Peru, Iquitos, Belen, Itaya	54	4*x*	MH177321	MH177365	MH198375	MH177409	MK809536-MK809541
	C.-I Peng 21936	Vietnam, Long Son	78	4*x*					MK820724-MK820730
L. peploides (Kunth) P. H. Raven	GenBank				KX154654		KX168181; AY905473		AJ550746

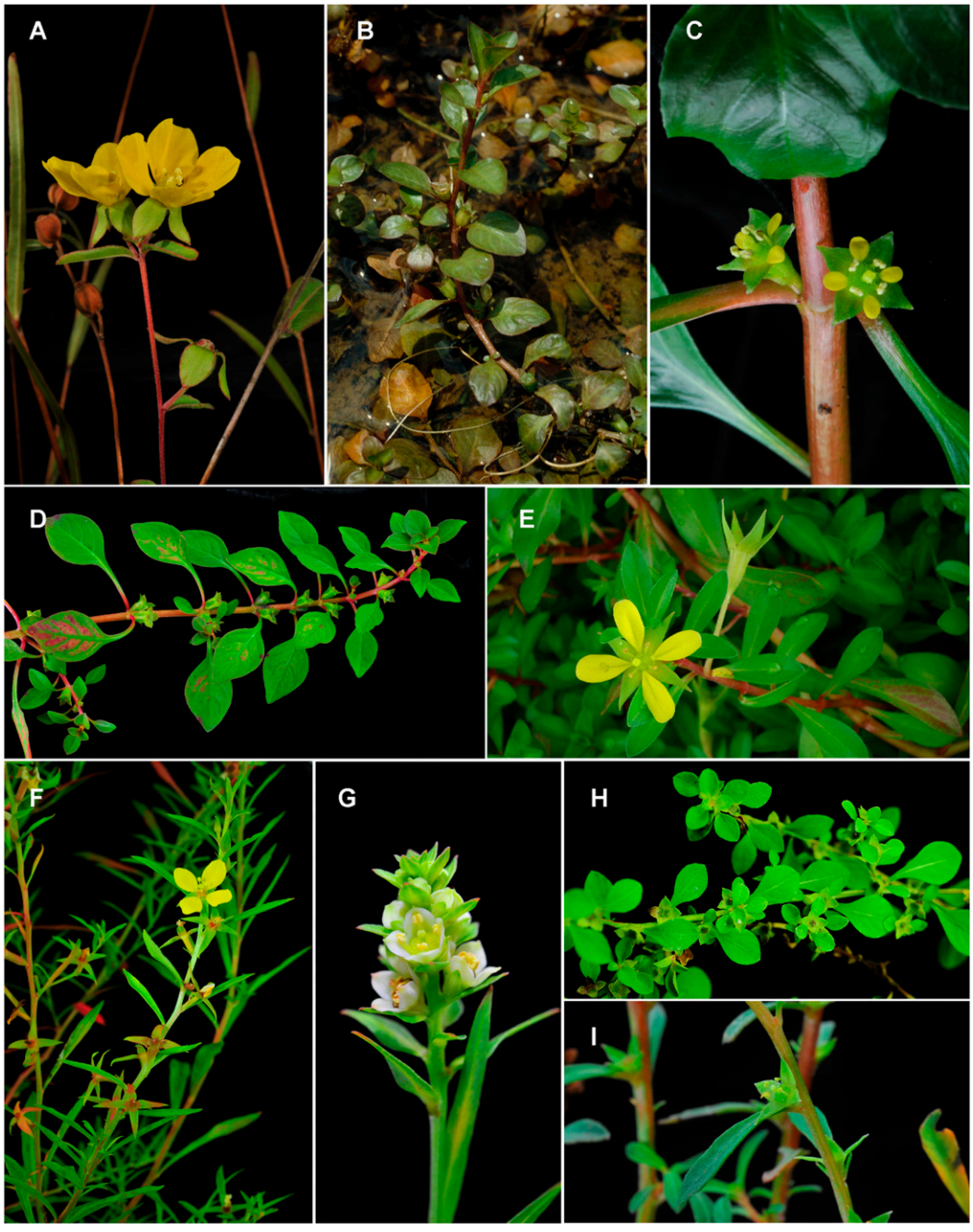

Figure 1. Representatives of the North Temperate haplostemonous *Ludwigia* L. group. —A. *Ludwigia maritima* R. M. Harper (section *Ludwigia*). —B. *Ludwigia ovalis* Miq. (section *Miquelia* P. H. Raven). —C. *Ludwigia repens* J. R. Forst. (section *Isnardia* (L.) W. L. Wagner & Hoch). —D. *Ludwigia palustris* (L.) Elliott (section *Isnardia*). —E. *Ludwigia brevipes* (Long) Eames (section *Isnardia*). —F. *Ludwigia linifolia* Poir. (section *Microcarpium* Munz). —G. *Ludwigia suffruticosa* Walter (section *Microcarpium*). —H. *Ludwigia microcarpa* Michx. (section *Microcarpium*). —I. *Ludwigia curtissii* Chapm. (section *Microcarpium*).

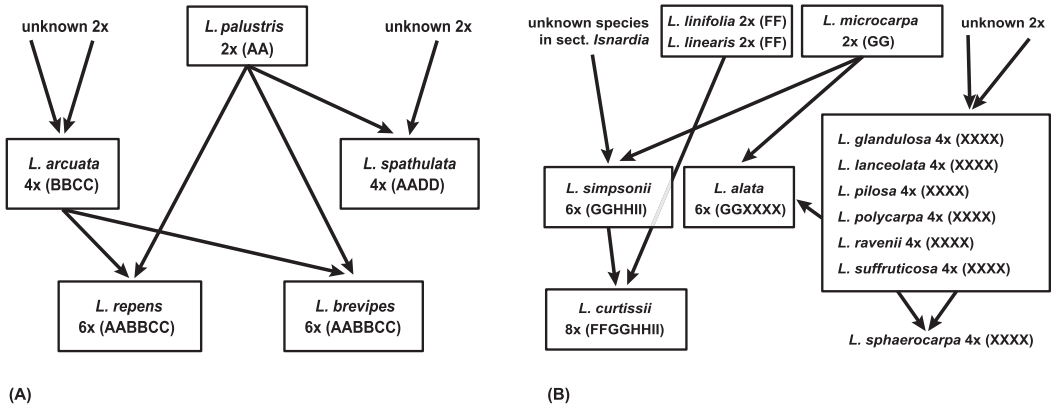

Figure 2. Hypotheses of the reticulate and polyploid evolution of *Ludwigia* L. sect. *Isnardia* (L.) W. L. Wagner & Hoch (A) and section *Microcarpium* Munz (B) redrawn based on Peng (1988, 1989) and Peng et al. (2005). Note that the genome of *L. polycarpa* Short & R. Peter was inferred from phylogenies of Liu et al. (2017).

likely resulted from hybridization between *L. microcarpa* (GG) and the tetraploid (XXXX). The Cuban endemic *L. stricta* (C. Wright ex Griseb.) C. Wright is a diploid closely related to *L. linifolia*, though its genome has not been characterized. Despite some morphological differences, there is considerable similarity between sections *Isnardia* and *Microcarpium* in overall morphology and distributional range (Wagner et al., 2007), with frequent intersectional hybridizations reported (Raven & Tai, 1979; Peng, 1988, 1989; Peng et al., 2005). Hypotheses of the reticulate evolution of *Ludwigia* sect. *Microcarpium* proposed in Peng (1988, 1989) and section *Isnardia* in Peng et al. (2005) are summarized in Figure 2.

Recent phylogenetic study focused on *Ludwigia* strongly (chloroplast [cp] DNA) or moderately (nuclear [nr] DNA) supports that section *Ludwigia* and the *Microcarpium* complex are sister clades (Liu et al., 2017). Liu et al. (2017) also found that neither section *Isnardia* nor section *Microcarpium* is monophyletic. Placement of section *Miquelia* was unresolved, either within the *Isnardia–Microcarpium* clade (nrDNA) or sister to it (cpDNA; Liu et al., 2017), suggesting a possible hybrid origin of *L. ovalis* (e.g., Small et al., 2004; Govindarajulu et al., 2015; Gong et al., 2017).

Several phylogenetic studies have tested Peng's hypotheses (Fig. 2) partially (Hung et al., 2009; Hsu, 2010; Liu et al., 2017). Based on cp and ITS sequences, these studies suggested that *Ludwigia palustris* (AA) contributed as paternal parent to the origin of *L. spathulata* (AADD) and as maternal parent to the origins of *L. polycarpa* (AAXX) (Liu et al., 2017) and *L. brevipes* (AABBCC) (Hung et al., 2009). Liu et al. (2017) also suggested that the tetraploid *L. polycarpa* (section *Microcarpium*) possesses the A genome (Fig. 2), and that *L. arcuata* (BBCC) is likely the maternal genome donor for *L. brevipes* (AABBCC) and possibly also for *L. repens* (AABBCC) (Hung et al., 2009). Moreover, these analyses verified that *L. microcarpa* (GG) contributed to the origin of *L. simpsonii* (GGHHII) (Hsu, 2010; Liu et al., 2017), that *L. simpsonii* was a parental donor to *L. curtissii* (FFGGHHII) (Hsu, 2010), and that the tetraploid *L. lanceolata* or *L. suffruticosa* likely contributed to the origin of the hexaploid *L. alata* (GGXXXX) (Liu et al., 2017). However, none of these studies have sampled adequately to test Peng's hypotheses (Fig. 2; Peng, 1988, 1989; Peng et al., 2005) and completely resolve relationships within the NTH *Ludwigia* group.

To disentangle the reticulate evolution of the NTH *Ludwigia* group, we used DNA sequences of the biparentally inherited nuclear single-copy region, *PgiC* exon11–exon16, and four maternally inherited chloroplast regions (*rpL16*, *rpoB-trnC*, *trnL-trnF*, and *ycf6-psbM*). By integrating our results with earlier biosystematic studies (Peng, 1988, 1989; Peng et al., 2005), evolutionary history of this complicated group is elucidated. Based on our result, we propose to revise the taxonomy of the NTH clade and infer the biogeographic origin of the East Asian species of the NTH *Ludwigia*.

Materials and Methods

Sampling

We sampled all species of the NTH *Ludwigia* group except for the Cuban endemic species *L. stricta*, which is very similar to *L. linifolia* (Peng, 1988, 1989). We also sampled two outgroups—*L. adscendens* (L.) H. Hara and *L. peploides* (Kunth) P. H. Raven—from the sister clade (clade B in Liu et al., 2017) of the NTH *Ludwigia* clade to root our phylogeny. Living plants or seeds of most species were collected from natural populations and grown at the Academia Sinica greenhouse. Full voucher

information is provided in Table 1. The DNA sequences of *L. peploides* were downloaded from the National Center for Biotechnology Information (NCBI) GenBank.

CHROMOSOME CYTOLOGY

To confirm species identification and ploidy level, root tips from living plants of the NTH *Ludwigia* clade (Table 1) at the Academia Sinica greenhouse were examined to count somatic chromosomes. The procedures of chromosome observations followed Kono et al. (2012) and Peng et al. (2014). Root tips were pretreated with 2 mM 8-hydroxyquinoline solution at 15°–18°C for 6 to 8 hours and then fixed overnight in Carnoy's fluid (95% ethanol and acetic acid in the ratio of 3:1) at 4°C. They were then macerated with an enzyme mixture, including 2% Cellulase Onozuka R-10 (Yakult Honsha, Tokyo, Japan) and 1% Pectolyase (Sigma, St. Louis, Missouri, U.S.A.) at 37°C for about 1 hour. Root tips were then cut and squashed in 45% acetic acid. Later, 2% Giemsa solution (Merck, Darmstadt, Germany) was applied to stained root tips at room temperature for about 10 minutes. Cells at mitotic metaphase were examined and photographed with a Zeiss Axio Imager.A1 (Zeiss, Oberkochen, Germany) and AxioCam MRm monochrome digital camera (Zeiss).

DNA REGION SELECTION, SEQUENCING, AND CLONING

Genomic DNA was extracted from fresh leaves of living collections using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Four chloroplast noncoding regions (*rpL16*, *rpoB-trnC*, *trnL-trnF*, and *ycf6-psbM*) were selected and successfully amplified for all samples. These chloroplast regions have been used in previous phylogenetic studies for various taxa (e.g., Taberlet et al., 1991; Shaw et al., 2005; Chung et al., 2014). For nuclear regions, it is known that multiple-copy regions such as ITS are often unable to resolve polyploid evolution because of their heterozygosity and paralogy (Baldwin et al., 1995; Soltis & Soltis, 1998; Alvarez & Wendel, 2003). Here, a single-copy nuclear region, *PgiC* exon11–exon16, was chosen and applied for all *Ludwigia* samples.

The *PgiC* regions have provided insights into the evolutionary histories of plant groups that include polyploids (e.g., Ford & Gottlieb, 1999; Ford et al., 2006; Juslén et al., 2011), and in particular have elucidated tribal and sectional relationships in Onagraceae (Gottlieb & Ford, 1996; Ford & Gottlieb, 2007). Earlier studies revealed that *PgiC* appears as a single-copy gene in some Onagraceae lineages (e.g., *Circaea cordata* Royle, *Fuchsia cylindracea* Lindl., and *Hauya heydeana* Donn. Sm.), but has two copies in others (e.g., some sections in *Clarkia* Pursh, *Epilobium canum* (Greene) P. H. Raven, and *E. brachycarpum* C. Presl) (Thomas et al., 1993; Ford & Gottlieb, 2002, 2007). The copy number of *PgiC* regions in *Ludwigia* species was unknown. In this study, we modified the universal primers for *PgiC* exon11–exon16 region developed by Ford et al. (2006), screened sequences from Ford and Gottlieb's (2007) study, and then replaced some degenerate sites with the DNA bases that are consistent in Onagraceae. Our preliminary results with the modified primers show that *PgiC* exon11–exon16 regions of diploid NTH *Ludwigia* species do not contain a polymorphic site (Supplementary Appendix S1)—that is, diploid NTH *Ludwigia* species have only one single copy of *PgiC* exon11–exon16 region. The modified primers are reported in Table 2.

The polymerase chain reaction (PCR) amplifications were conducted with a mixture of 0.5 μL genomic DNA, 1 μL forward primer (10 mM), 1 μL reverse primer (10 mM), 10 μL Taq DNA Polymerase Master Mix Red (Ampliqon, Copenhagen, Denmark), and 7.5 μL deionized water. The optimal thermal cycling conditions for the studied regions are provided in Supplementary Table S1.

The PCR products of the chloroplast regions were purified with the PCR Advanced Clean Up (Viogene BioTek Corp., New Taipei City, Taiwan) and sequenced commercially with the Genomics BioSci & Tech. Ltd. (New Taipei City, Taiwan). For polyploid *Ludwigia* samples, the purified PCR products of *PgiC* region were cloned using the pGEM-T Vector Systems (Promega, Madison, Wisconsin, U.S.A.). Positive colonies were screened using the T7 and SP6 primers and a PCR reaction with our optimal thermal cycling conditions (Supplementary Table S1). Previous hypotheses (Peng, 1988, 1989; Peng et al., 2005) suggested that two genomes were involved in the origin of each tetraploid NTH *Ludwigia* taxon (Fig. 2). To ascertain the proposition, eight positive clones were sequenced for each tetraploid sample. Similar criteria were applied to other polyploids: 10 to 12 clones were selected for hexaploids, and 16 to 20 clones for octoploids. Selected colonies were purified and sequenced. Contigs were assembled in SeqMan II software 5.0 (DNA Star, Inc., Madison, Wisconsin, U.S.A.). Newly generated sequences have been submitted to GenBank, and accession numbers are reported in Table 1.

PHYLOGENY RECONSTRUCTION

Sequences were aligned with MAFFT 7 (Katoh et al., 2002; Katoh & Standley, 2013) and adjusted in Mesquite 3.51 (Maddison & Maddison, 2018), if needed. Previous study suggests a lack of recombination in *Ludwigia* plastomes based on genome comparisons (Liu et al., 2017). Hence, in the present study, the alignments of the four chloroplast regions were concatenated for phylogenetic analyses.

Table 2. Primers used in this study and their origins.

DNA region	Primers	Primer sequence (5′ →3′)	Reference
rpL16	F	GCTATGCTTAGTGTGTGACTCG	Chung et al. (2014)
	R	CGTCCYGCTTCTATTTGTCTAG	
rpoB-trnC	rpoB	CKACAAAAYCCYTCRAATTG	Shaw et al. (2005)
	trnC(GCA)-R	CACCCRGATTYGAACTGGGG	
trnL-trnF	trnL5′(UAA)F (TabC)	CGAAATCGGTAGACGCTACG	Taberlet et al. (1991); Shaw et al. (2005)
	trnF(GAA) (TabF)	ATTTGAACTGGTGACACGAG	
ycf6-psbM	ycf6F	ATGGATATAGTAAGTCTYGCTTGGGC	Shaw et al. (2005)
	psbMR	ATGGAAGTAAATATTCTYGCATTTATTGCT	
PgiC	AA11F-2	TTTGCNTTYTGGGATTGGGT	modified from *PgiC* universal primers from Ford et al. (2006)
	AA16R-2	CCCTTGCCRTTRCTCTCCAT	

Maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) algorithms were applied for chloroplast and *PgiC* regions. In MP analyses, 200 searches were conducted in PAUPRat (Sikes & Lewis, 2001), and a strict consensus tree was generated from the most parsimonious trees for each dataset. To estimate the homoplasies on MP trees, the consistency index (CI) and retention index (RI) were calculated in Mesquite 3.51 (Maddison & Maddison, 2018) and the rescaled consistency (RC) and homoplasy index (HI) were also computed. The homoplasy and comparative statistics of MP analyses are available in Table 3. The best-fit nucleotide substitution models for chloroplast and *PgiC* regions were estimated with the Akaike information criterion (AIC) in jModeltest 2.1.8 (Darriba et al., 2012) and then applied in ML and BI trees reconstructions. The best-fit models are shown in Table 3. The ML trees were reconstructed with RAxML 8.2.10 (Stamatakis, 2014) on the CIPRES Science Gateway 3.3 (Miller et al., 2010) with 10 tree searches and 1000 rapid bootstrap (BS) procedures. Bayesian analyses were performed in MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001; Ronquist et al., 2012) on the CIPRES Science Gateway 3.3 as well as with two independent Markov chain Monte Carlo (MCMC) runs. Each run began from a random tree and four simultaneous chains set at default temperature (Huelsenbeck & Ronquist, 2001). Each run was performed for 5×10^6 generations, and one tree was sampled every 1000th generation. The initial 25% of trees were ignored as burn-in. The remaining trees were summed to generate a 50% majority rule consensus tree and to estimate the posterior probabilities (PP) on each branch. All trees were viewed with FigTree 1.4.3 (Rambaut, 2014).

BIOGEOGRAPHIC INFERENCE

To infer the biogeographic origin of *Ludwigia ovalis*, the only East Asian species of the NTH *Ludwigia*, we first reconstructed the representative trees based on

Table 3. Comparative statistics for maximum parsimony analyses of the North Temperate haplostemonous *Ludwigia* L. and the best-fit nucleotide substitution models for the analyzed datasets.

Analyzed dataset	The best-fit substitution models	Number of sequence/ Ingroup spp. #/Number of ingroup sequence/Number of outgroup sequence	Length in alignments (bp)	Number of parsimony informative characters (% of parsimony informative characters)	Number of uninformative variable characters	CI/ RI/ RC/ HI
cp tree	GTR+I+G	45/ 23/ 43/ 2	4371	131 (3.00%)	88	0.8604/ 0.9454/ 0.8134/ 0.1396
PgiC gene tree	HKY+I+G	37/ 23/ 362/ 14	1118	349 (31.22%)	200	0.6282/ 0.9372/ 0.5887/ 0.3718

CI, consistency index; cp, chloroplast; HI, homoplasy index; RC, rescaled consistency index; RI, retention index.

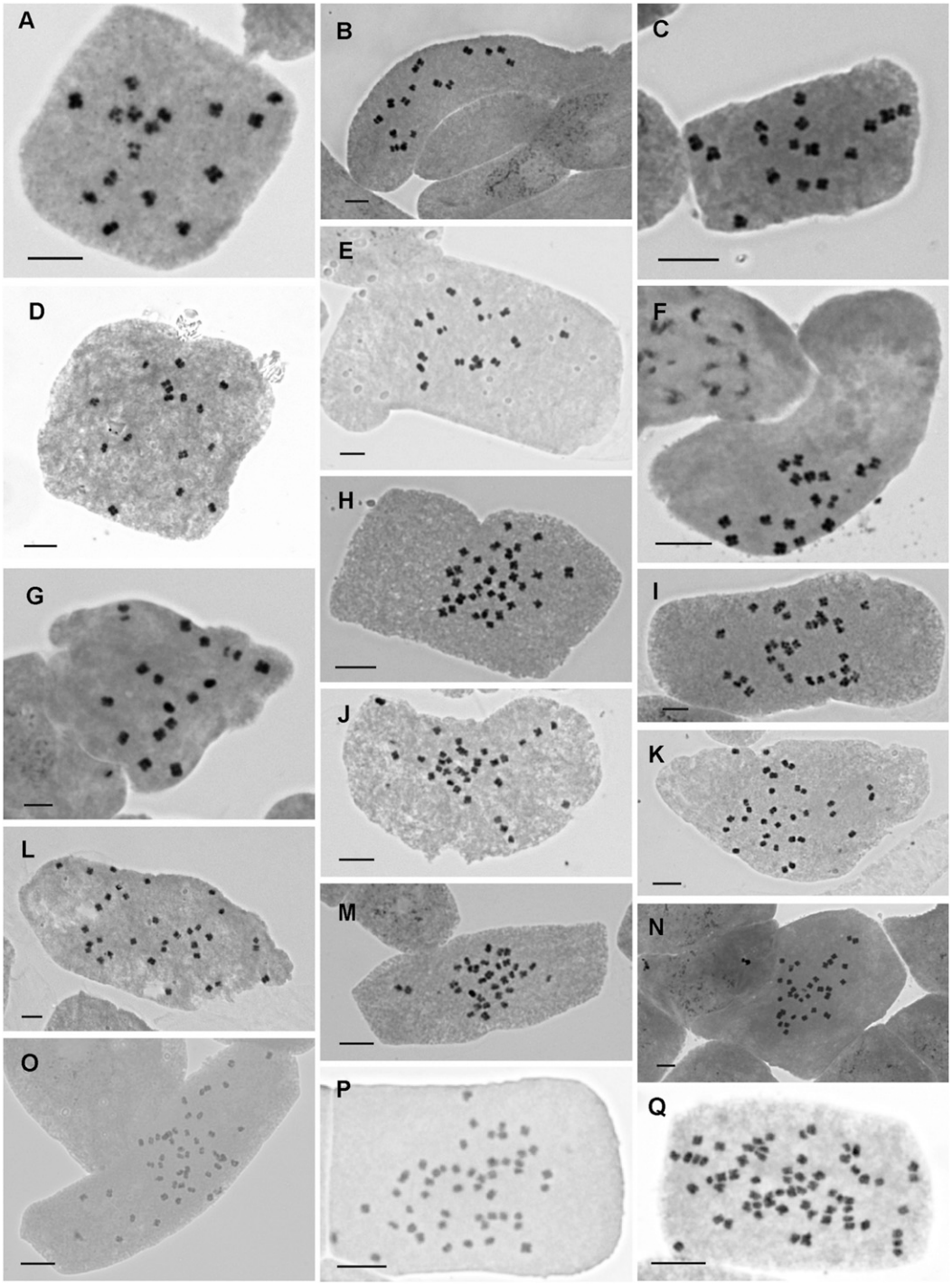

Figure 3. Mitotic chromosomes of selected taxa of the North Temperate haplostemonous *Ludwigia* L. group. —A. *Ludwigia alternifolia* L., $2n = 16$. —B. *Ludwigia hirtella* Raf., $2n = 16$. —C. *Ludwigia maritima* R. M. Harper, $2n = 16$. —D. *Ludwigia virgata* Michx., $2n = 16$. —E. *Ludwigia linifolia* Poir., $2n = 16$. —F. *Ludwigia microcarpa* Michx., $2n = 16$. —G. *Ludwigia linearis* Walter, $2n = 16$. —H. *Ludwigia sphaerocarpa* Elliott, $2n = 32$. —I. *Ludwigia glandulosa* Walter, $2n = 32$. —J. *Ludwigia lanceolata* Elliott, $2n = 32$. —K. *Ludwigia ravenii* C.-I Peng, $2n = 32$. —L. *Ludwigia pilosa* Walter, $2n = 32$. —M. *Ludwigia polycarpa* Short & R. Peter, $2n = 32$. —N. *Ludwigia suffruticosa* Walter, $2n = 32$. —O. *Ludwigia alata* Elliott, $2n = 48$. —P. *Ludwigia repens* J. R. Forst., $2n = 48$. —Q. *Ludwigia curtissii* Chapm., $2n = 64$. Scale bar = 5 μm.

selected *PgiC* clones representing all genomes of all studied *Ludwigia* taxa and outgroups. Both ML and BI algorithms described in the previous section (Phylogeny Reconstruction) were applied to generate the representative trees. We defined six geographic areas for biogeographic inferences: (1) North America, (2) South America, (3) Africa, (4) Europe, (5) Asia, and (6) Pacific. The ancestral area was inferred using Mesquite 3.51 (Maddison & Maddison, 2018). Both the unordered parsimony and likelihood approach with Mk1 model were performed. For likelihood analysis, the polymorphic states were treated as uncertain.

Results

Chromosome cytology

Mitotic chromosomes of the NTH *Ludwigia* samples are shown in Figure 3, and chromosome numbers are reported in Table 1. Our chromosome counts are consistent with earlier cytological studies of *Ludwigia* (Raven & Tai, 1979; Peng, 1988; Peng et al., 2005), supporting our species identification and ploidy determination.

Phylogeny reconstruction

For chloroplast regions, our Shimodaira–Hasegawa (SH) tests indicate that the topologies of MP, ML, and BI trees based on the concatenated data set are not inconsistent (P values > 0.05 for all pairwise comparisons; Supplementary Table S2). Only the ML tree is shown here (Fig. 4, Supplementary Appendix S2).

Our cp tree shows that section *Ludwigia* and the *Microcarpium* complex are both monophyletic (Fig. 4). Within the *Microcarpium* complex, four clades are present, including *L. ovalis*, clade A^{cp}, clade F^{cp}, and clade G^{cp} (Fig. 4). The latter three clades are so named based on genome designation (Fig. 2) of the diploid species present in the clade. *Ludwigia ovalis* is placed with good support values (PP = 0.92, BS = 91) as a branch sister to a clade of sections *Isnardia* and *Microcarpium* (clades $A^{cp} + G^{cp} + F^{cp}$), neither of which is monophyletic. Because the chloroplast genome is maternally inherited in Onagraceae (Zhang et al., 2003), the cp tree can be used to infer the maternal genome of the polyploid NTH *Ludwigia* taxa by comparing with hypotheses outlined in Figure 2.

Clade F^{cp} is composed of diploid *Ludwigia linifolia* (FF) and two accessions of *L. linearis* (FF). Clade A^{cp} (PP = 0.99, BS = 100) includes the diploid *L. palustris* (AA) of section *Isnardia* and all tetraploids of section *Microcarpium* (XXXX), suggesting that these tetraploids have genome A or a genome closely related to A transmitted from the maternal parent (i.e., AAXX). Of the two accessions sampled for *L. sphaerocarpa*, *J. R. Abbott 23370* (12 + 61) is placed in the same clade with *L. lanceolata*, while *Anderson s.n.* (81) is placed closer to *L. suffruticosa* (Fig. 4).

Clade G^{cp} includes the diploid *Ludwigia microcarpa* (GG) of section *Microcarpium* and all remaining polyploids of the *Microcarpium* complex. The placement of the tetraploid *L. spathulata* (AADD) of section *Isnardia* in clade G^{cp} suggests that its maternal parent is more closely related to genome G, not genome A. The placement of *L. arcuata* in a well-supported but unresolved clade (PP = 1.00, BS = 98) with the two hexaploids of section *Isnardia* (i.e., *L. brevipes* [AABBCC] and *L. repens* [AABBCC]) suggests that *L. arcuata* is the maternal parent of the hexaploids, given that those hexaploids were hypothesized to have originated from hybridization (Fig. 2A) between *L. palustris* (AA) and *L. arcuata* (BBCC). The placement of *L. alata* (GGXXXX) in clade G^{cp} also suggests that its G genome was inherited maternally, given that *L. alata* was hypothesized to have originated through hybridization between *L. microcarpa* and the tetraploids (XXXX) of section *Microcarpium* (Fig. 2B). The placement of the hexaploid *L. simpsonii* (GGHHII) and octoploid *L. curtissii* (FFGGHHII) of section *Microcarpium* in a strongly supported clade suggests that *L. simpsonii* is the maternal parent of *L. curtissii* (Fig. 2B). Given Peng's (1989) proposition that section *Isnardia* likely contributed genomes to *L. simpsonii*, the placement of *L. curtissii–L. simpsonii* and *L. arcuata–L. brevipes–L. repens* in the same clade (PP = 1.00, BS = 96) suggests that the tetraploid *L. arcuata* or a species similar to it might be the maternal parent of *L. simpsonii* (Fig. 2B).

For the *PgiC* region, a total of 405 positive clones were sequenced. Poorly assembled and obviously artifactual sequences likely resulting from PCR error were removed. Finally, 376 clones representing 23 NTH *Ludwigia* species (46 samples) and *L. adscendens* (two samples) were used in this study. The SH tests show that the ML and BI trees based on *PgiC* region are congruent in their topologies (P value > 0.05; Supplementary Table S2), whereas the MP tree has a significantly different topology (P values < 0.05; Supplementary Table S2). The SH test results are not unexpected because both the ML and BI trees were reconstructed using the model algorithms and the MP tree was generated using a nonmodel algorithm. Here, only the ML tree (Supplementary Fig. S1) is presented.

Our *PgiC* tree (Supplementary Fig. S1) shows that the clones from each *Ludwigia* taxon are clustered into the expected number of clades corresponding to their respective ploidy levels except for *L. sphaerocarpa* of which four copies are detected for both accessions (see below). So the clones from a tetraploid are grouped into the two clades; the clones from a hexaploid were grouped into three clades; and the clones from an

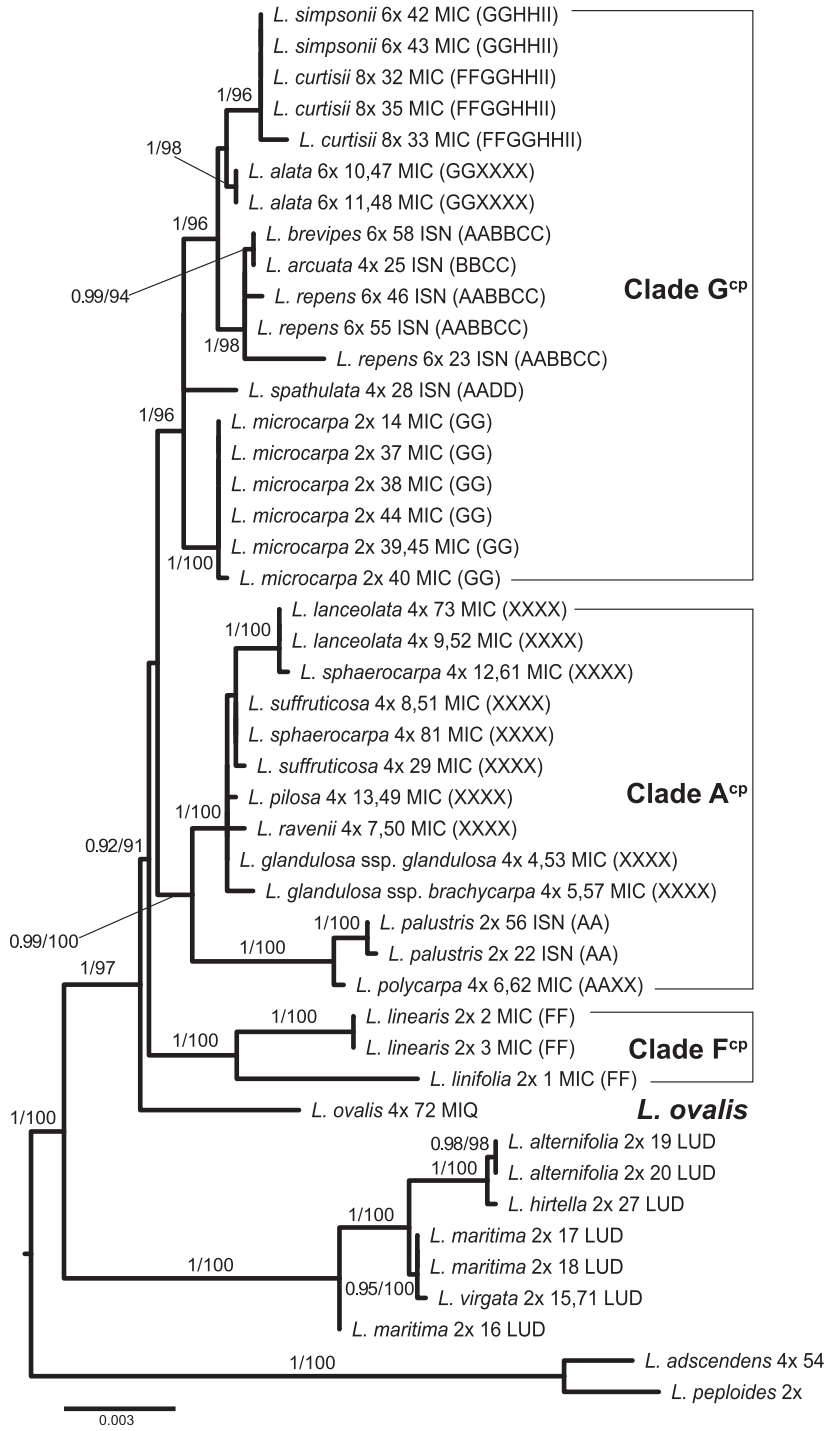

Figure 4. The maximum likelihood tree of North Temperate haplostemonous *Ludwigia* L. based on four chloroplast regions (*rpL16*, *trnL-trnF*, *ycf6-psbM*, and *rpoB-trnC*). Numbers on the branches show Bayesian posterior probabilities/bootstrap percentages. Ploidy level, sample number (see Table 1), section code (see Table 1), and genome hypothesized in earlier studies (see Fig. 2) are provided for each sequence. The scale bar indicates the branch length.

octoploid into four clades. To facilitate the following discussion, all clones (branches) of a genome clade from the same taxa were collapsed. For the cases where clones from two taxa are nested in the same clade, all branches of the nested clade were collapsed into one branch. The collapsed *PgiC* tree is presented in Figure 5.

The collapsed *PgiC* tree (Fig. 5) agrees with the cp tree (Fig. 4) in supporting section *Ludwigia* and the *Microcarpium* complex as monophyletic sister clades. Within the *Microcarpium* complex, eight clades are identified with strong branch support values (PP = 1.00, BS ≥ 75; Fig. 5); these clades are named A, B/H, C/I, D/G, F_1, F_2, M_1, and M_2 based on presence of the corresponding diploid genome and/or to match with hypotheses of Peng (1988, 1989) and Peng et al. (2005) illustrated in Figure 2. Only clades A, D/G, F_1, and F_2 include diploid species. Among the eight clades, clades A and C/I are sister groups (PP = 1.00, BS = 82), whereas clades B/H, F_1, and F_2 form a monophyletic but unresolved group (PP = 1.00, BS = 97). Clade M_2 of *L. ovalis* forms a separate branch of the basal polytomy of the *Microcarpium* complex, whereas clade M_1 of *L. ovalis* is a separate branch sister to clade A + clade C/I.

Among the eight clades of the *Microcarpium* complex, clades M_1 and M_2 contain only *Ludwigia ovalis* (section *Miquelia*), supporting the species as an allotetraploid. However, since clades M_1 and M_2 include no extant diploids, both diploid ancestors of *L. ovalis* had likely gone extinct.

Clade A is so named for the presence of the diploid *Ludwigia palustris* (AA). In addition to *L. palustris*, clade A also includes all tetraploids (XXXX) and the hexaploid *L. alata* (GGXXXX) of section *Microcarpium*, and the tetraploid *L. arcuata* (BBCC) and hexaploids *L. brevipes* (AABBCC) and *L. repens* (AABBCC) of section *Isnardia* (Fig. 5). Because our cpDNA tree (Fig. 4) has inferred *L. arcuata* (BBCC) as the maternal parent of both *L. brevipes* (AABBCC) and *L. repens* (AABBCC), the presence of these two hexaploids in clade A suggests that their paternal parent contributed genome A. Similarly, *L. spathulata* (AADD) received genome A through its paternal parent, given that it inherited through its maternal parent a genome closer to that of *L. microcarpa* (GG), as inferred from the cpDNA tree (Fig. 4). The presence of all tetraploids of section *Microcarpium* in clade A is consistent with the cpDNA tree (Fig. 4), while the presence of *L. alata* (GGXXXX) in this clade further resolves its genome formula from "GGXXXX" to "AAGGXX."

Clade D/G is so named because of the presence of the diploid *Ludwigia microcarpa* (GG) and the tetraploid *L. spathulata* (AADD); as just noted, the other genome of *L. spathulata* has been identified in clade A of the *PgiC* tree (Fig. 5). The presence of both *L. microcarpa* (GG) and *L. spathulata* (AADD) in clade D/G is also congruent with our inference (Fig. 4) that the maternal genome of *L. spathulata* and genome G are closely related. Clade D/G also includes the hexaploids (*L. alata* [AAGGXX] and *L. simpsonii* [GGHHII]) and the octoploid *L. curtissii* (FFGGHHII) of section *Microcarpium* (Fig. 5), consistent with Peng's (1988) hypothesis (Fig. 2) and inference from our cpDNA tree (Fig. 4).

Although both *Ludwigia linifolia* (clade F_1) and *L. linearis* (clade F_2) were hypothesized to have the F genome, our *PgiC* tree indicates that they do not form a single clade, and that *L. linifolia* (clade F_1), not *L. linearis* (clade F_2), contributed its F_1 genome to the octoploid *L. curtissii* (FFGGHHII) as the paternal parent (Fig. 5); the cpDNA tree has identified *L. simpsonii* (GGHHII) as the maternal parent (Fig. 4).

Clade B/H and clade C/I do not include any diploid taxa but are so named because of the presence in both tetraploid *Ludwigia arcuata* (BBCC) and hexaploid *L. simpsonii* (GGHHII), given genome G of the latter has already been identified in clade D/G (Fig. 5). Clade B/H includes all polyploid taxa of the *Microcarpium* complex except for *L. spathulata* (AADD), indicating the presence of the B/H genome in all other polyploids (Fig. 5). Because our cpDNA tree has inferred that the tetraploids of section *Microcarpium* inherited genome A maternally (Fig. 4), the *PgiC* tree suggests that these tetraploids inherited their B/H genome paternally (i.e., AABB). Consequently, the hexaploid *L. alata* (AAGGXX) can now be designated AABBGG (≡ AABBDD). In both clades B/H and C/I, *L. simpsonii–L. curtissii* is sister to *L. arcuata–L. brevipes–L. repens* (Fig. 5), consistent with their morphological similarity (Peng, 1989) and our inference from the cpDNA tree that *L. arcuata* is likely the maternal parent of *L. simpsonii* (Fig. 4), indicating that genomes B and C are similar to H and I, respectively. By substituting H with B and I with C, the genome complement of *L. simpsonii* and *L. curtissii* should be BBCCDD and BBCCDDFF, respectively.

Both accessions of *Ludwigia sphaerocarpa* (*Anderson s.n.* [81] and *J. R. Abbott 23370* [12 + 61], Table 1) possess four copies of *PgiC* genes, two in clade A and two in clade B/H (Fig. 5, Supplementary Fig. S1). In both clades, the two copies of *Anderson s.n.* (81) are grouped either by themselves or with a copy of *J. R. Abbott 23370*, but the second copy of *J. R. Abbott 23370* is grouped with *L. lanceolata* (Fig. 5, Supplementary Fig. S1). The presence of two copies of genome A and genome B/H is congruent with Peng's (1988) hypothesis that *L. sphaerocarpa* originated after hybridization between the interfertile tetraploids. The different genome components in *J. R. Abbott 23370* and *Anderson s.n.* are also consistent with Peng's (1988) proposition that *L. sphaerocarpa* might have multiple origins.

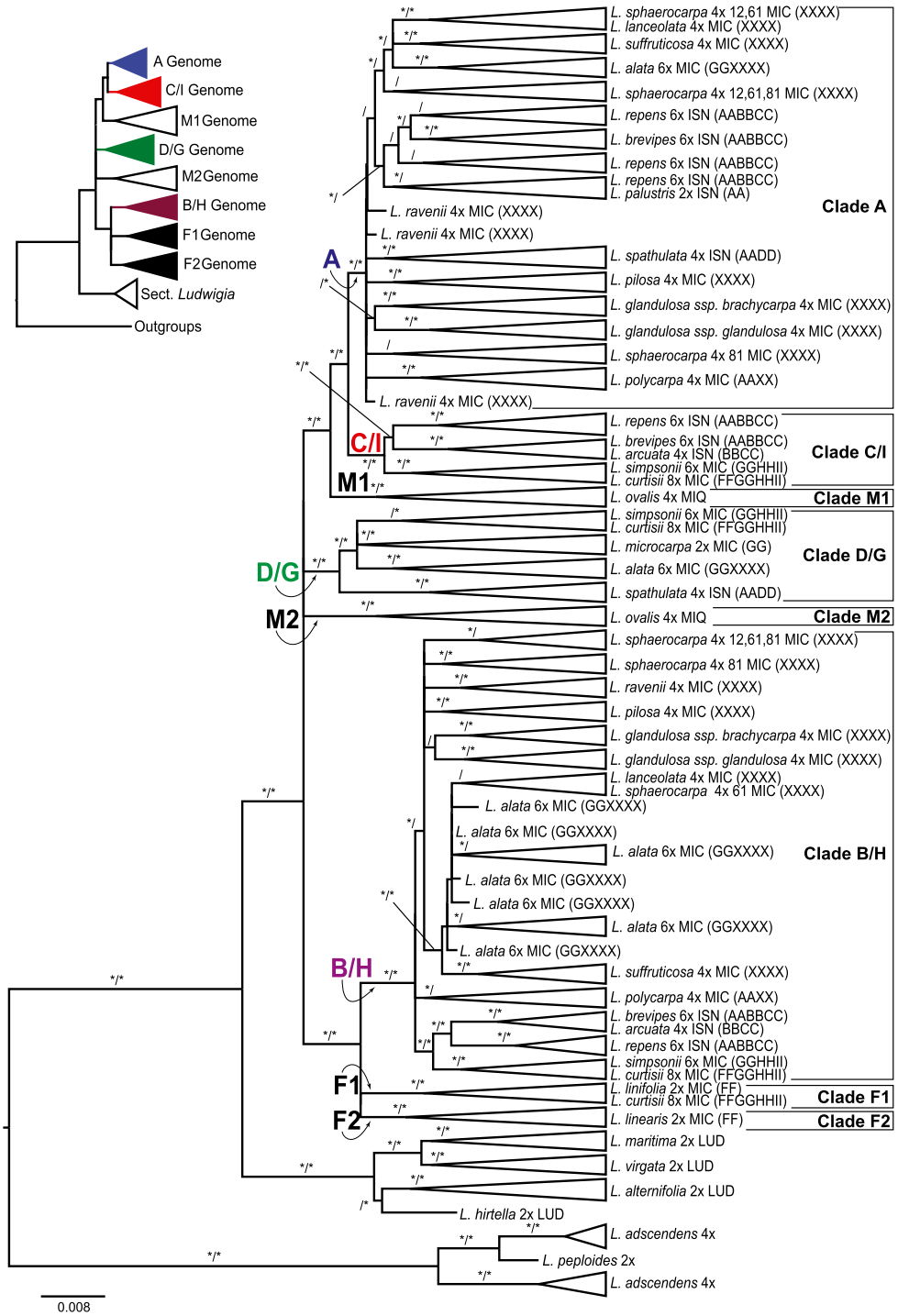

Figure 5. The collapsed *PgiC* maximum likelihood tree of the North Temperate haplostemonous *Ludwigia* L. clade. Uncollapsed tree is depicted in Supplementary Figure S1. Asterisks on the branches show Bayesian posterior probabilities > 0.9/bootstrap percentages > 70. Ploidy level, section code (see Table 1), and genome hypothesized in earlier studies (see Fig. 2) are provided for each terminal node. Sample numbers are included after ploidy levels only for *L. sphaerocarpa* Elliott (see Table 1). Letters in bold refer to genome designation. The scale bar indicates the branch length.

Since all seven tetraploids in section *Microcarpium* have both genomes A and B/H (Fig. 5), an additional phylogeny containing only these tetraploids was reconstructed by concatenating genome A and B/H sequences to understand their evolutionary relationships. This additional tree is shown in Supplementary Figure S2 (see also Supplementary Appendix S3).

BIOGEOGRAPHIC INFERENCE

The representative ML and BI trees were reconstructed based on 52 selected *PgiC* clones representing all genomes (A, B/H, C/I, D/G, F_1, F_2, M_1, and M_2) of all studied *Ludwigia* taxa and three clones from outgroups (two from *L. adscendens* and one from *L. peploides*). Only the representative ML tree is shown here (Fig. 6, Supplementary Appendix S4). The results of both parsimony and likelihood approaches of ancestral area reconstruction using Mesquite are consistent. However, because the likelihood approach is not capable of tracking polymorphic states, some nodes are left uncertain. Consequently, results of parsimony analyses are shown in the ML tree (Fig. 6). Analysis using Mesquite identifies North America as the ancestral area for the NTH *Ludwigia*, suggesting a dispersal event for the disjunct East Asian *L. ovalis*.

Discussion

PHYLOGENETIC RELATIONSHIP OF SECTION *LUDWIGIA*

Both the diploid section *Ludwigia* and the multiploid *Microcarpium* complex are well-supported monophyletic groups in both the cpDNA tree and *PgiC* tree (Figs. 4, 5), in accord with previous hypotheses (Raven & Tai, 1979; Wagner et al., 2007; Liu et al., 2017). Within section *Ludwigia*, the *PgiC* tree indicates that the clade of *L. alternifolia* L.–*L. hirtella* Raf. is sister to the clade of *L. maritima* R. M. Harper–*L. virgata* Michx., as predicted based on prior morphological and phylogenetic studies (Harper, 1904; Munz, 1965; Liu et al., 2017). This relationship is also consistent with the cpDNA tree (Fig. 4) except for one sample (*J. R. Abbott 23333* [16], Table 1) of *L. maritima* placed as the basal branch of section *Ludwigia*. More comprehensive sampling of *L. maritima* and *L. virgata* using additional DNA regions will be needed to clarify the delimitations and relationships of the two species.

RETICULATE EVOLUTION OF THE *MICROCARPIUM* COMPLEX

Within the *Microcarpium* complex, both cpDNA and *PgiC* trees (Figs. 4, 5) correspond well to Peng's (1988, 1989) and Peng et al.'s (2005) hypotheses of the relationships in sections *Isnardia* and *Microcarpium* (Fig. 2). Integrating Peng's cytological results with our trees, we propose a new model for the reticulate evolution in NTH *Ludwigia* (Fig. 7).

The *PgiC* tree indicates that the East Asian species *Ludwigia ovalis* (monospecific section *Miquelia*) is an allotetraploid consisting of genomes M_1 and M_2, concordant with results from Liu et al. (2017). In the *PgiC* tree (Fig. 5), genome M_2 appears as part of the basal polytomy of the *Microcarpium* complex, whereas genome M_1 is sister to the A–C/I clade. This relationship suggests that M_2 might be the maternal genome and M_1 the paternal genome of *L. ovalis* (Fig. 7), given *L. ovalis* is placed basal to the rest of the *Microcarpium* complex in the cpDNA tree (Fig. 4).

Both the cpDNA and *PgiC* trees indicate that neither section *Isnardia* nor section *Microcarpium* as currently delimited is a monophyletic group (Figs. 4, 5), congruent with the results of Liu et al. (2017). These results also support Peng's (1988) hypothesis (Fig. 2A) and further suggest that six of the seven tetraploid species in section *Microcarpium* (*Ludwigia glandulosa*, *L. lanceolata*, *L. pilosa*, *L. polycarpa*, *L. ravenii*, and *L. suffruticosa*) are the progeny of a common allotetraploid ancestor (AABB), which inherited maternal A and paternal B/H genomes (Fig. 6). However, our *PgiC* tree indicates that *L. sphaerocarpa* possesses four homeologs, two of clade A and two of clade B/H, respectively (Fig. 5, Supplementary Fig. S2). Based on the orientation of seed surface cells, Peng (1989) hypothesized that *L. sphaerocarpa* had a hybrid origin, likely resulting from hybridization between interfertile tetraploid species of section *Microcarpium*. Given its likely hybrid origin, the presence of four homeologs of *PgiC* in both accessions suggests that *L. sphaerocarpa* could be a homoploid hybrid species between two allotetraploid species of section *Microcarpium* (Ferguson & Sang, 2001). However, the two accessions of *L. sphaerocarpa* have different genome compliments (Fig. 5, Supplementary Fig. S2), suggesting that the species might have arisen from at least two hybridization events, one likely involving *L. lanceolata* (Levin, 2002; Barker et al., 2016).

Congruent with Peng's (1988) proposition, our *PgiC* tree suggests that hexaploid *Ludwigia alata* was likely derived following hybridization between a diploid *L. microcarpa* (section *Microcarpium*; D/G genome; ♀) and tetraploid species (AABB; ♂) closely related to *L. lanceolata*, *L. sphaerocarpa*, and *L. suffruticosa*. Our data also suggest that the other hexaploid of section *Microcarpium*, *L. simpsonii*, is an allohexaploid derived from hybridization between diploid *L. microcarpa* (♂) and an unidentified/extinct tetraploid ancestor (BBCC; ♀) closely related to *L. arcuata* of section *Isnardia*, supporting Peng's (1988) hypotheses based on cytology and morphology (Fig. 2A). The octoploid *L. curtissii* of section *Microcarpium* appears to be derived from

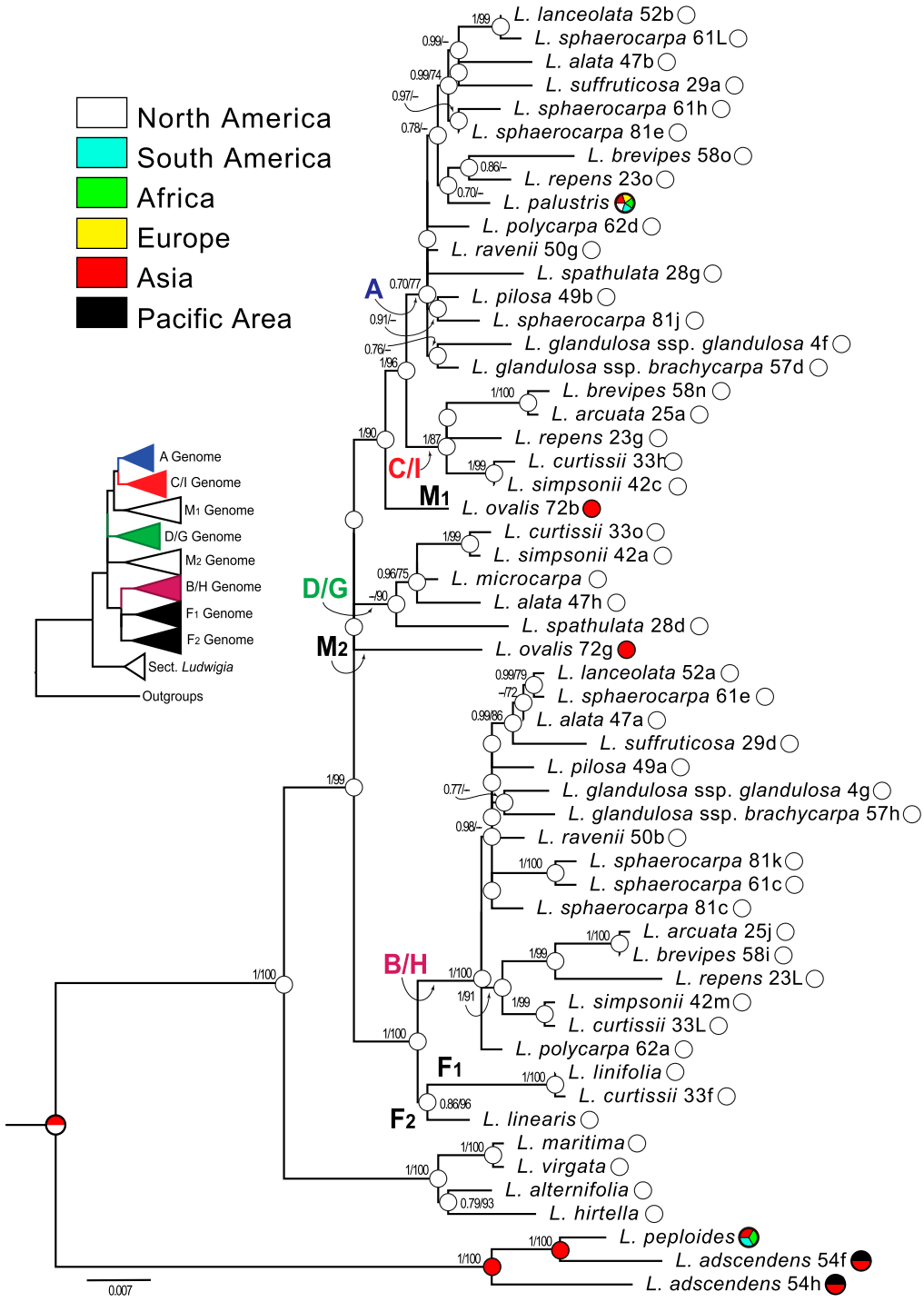

Figure 6. Ancestral area reconstruction using Mesquite. The scale bar indicates the branch length.

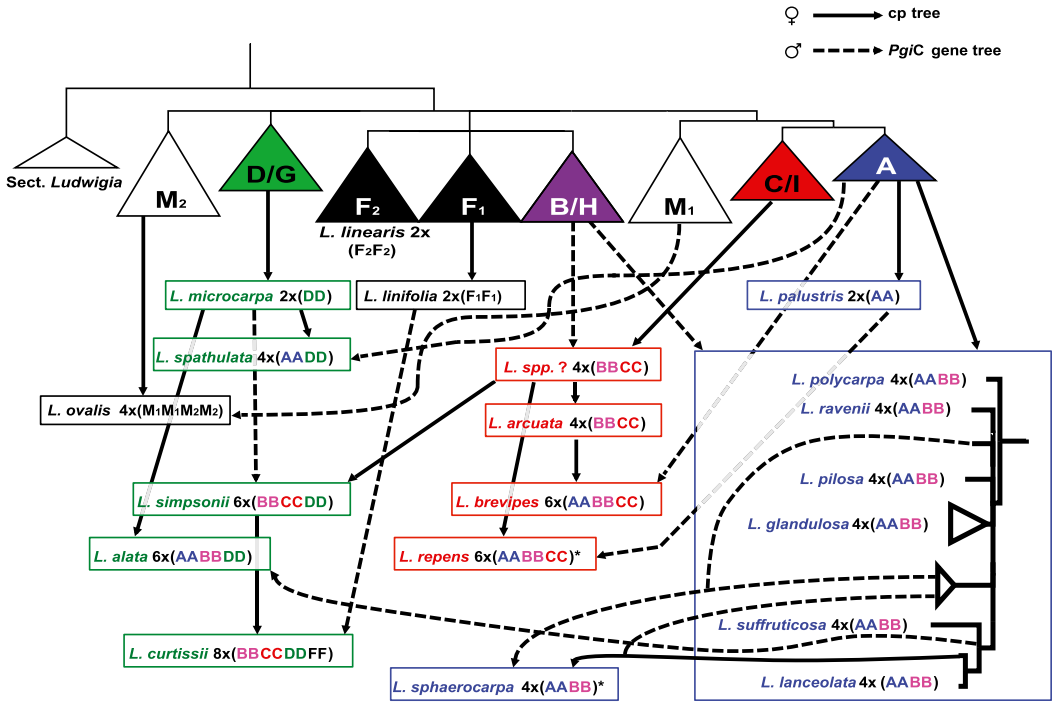

Figure 7. Reconstruction of the polyploid and reticulate evolution of the North Temperate haplostemonous *Ludwigia* L. clade based on the *PgiC* and chloroplast trees (Figs. 4, 5) and cytological and hybridization analyses of Peng (1988, 1989) and Peng et al. (2005). Backbone of tree is collapsed *PgiC* tree extracted from Figure 5, with genomes indicated in bold. Inset at right is extracted from Supplementary Figure S2, the *PgiC* tree based on genomes A and B for all tetraploid species in section *Microcarpium* Munz. Ploidy level and genome designation is included for each species. Asterisks indicate species with multiple origins (see text for details). Solid arrows connect species with maternal donors/genomes, and dashed arrows with paternal donors/genomes.

hybrids between *L. simpsonii* (6×; ♀) and *L. linifolia* (2×; genome F; ♂).

Our analyses also infer that the two tetraploids of section *Isnardia*—*Ludwigia spathulata* and *L. arcuata*—are derived from different genomes (Fig. 7), as proposed by Peng et al. (2005). *Ludwigia spathulata* derived from hybrids between *L. microcarpa* (2×; genome D/G; ♀) and an unidentified/extinct diploid ancestor with genome A (♂). *Ludwigia arcuata* derived from an unidentified/extinct tetraploid ancestor (BBCC) that also may have contributed to the origin of *L. simpsonii* (section *Microcarpium*) and *L. repens* (section *Isnardia*). The living diploid relatives of the genomes A, B/H, and C/I that contributed to these two tetraploids are either unidentified or extinct. In view of earlier intensive collecting and systematic studies on the NTH *Ludwigia* clade (Peng & Tobe, 1987; Peng, 1988, 1989; Tobe et al., 1988; Peng et al., 2005), it is more likely that the missing diploid progenitors had gone extinct rather than unidentified. In partial agreement with Peng et al. (2005) (Fig. 2B), our phylogenies suggest that the two hexaploid species of section *Isnardia* (*L. brevipes* and *L. repens*) evolved independently—but not from reciprocal directions (Peng et al., 2005)—though both of them inherited genomes A, B/H, and C/I. *Ludwigia brevipes* is likely a hybrid between the tetraploid *L. arcuata* (BBCC, ♀) and an extinct diploid ancestor with genome A (♂). Our analyses suggest that *L. repens* may have evolved multiple times, with the maternal donor probably the tetraploid ancestor (BBCC) that also gave rise to *L. arcuata* and *L. simpsonii*, and the paternal donor being either *L. palustris* (AA) or an extinct diploid closely related to *L. palustris*.

Even though our species-level phylogenies do not detect multiple origins for most polyploids in the NTH *Ludwigia* clade except for *L. sphaerocarpa* and *L. repens* (Fig. 7), it cannot be ruled out that other polyploids in the clade might also have multiple origins (Levin, 2002; Barker et al., 2016). Further analyses with additional samples of each polyploid species would be needed to test for other possible multiple origins.

While our analyses have thoroughly unraveled the reticulate evolution of the *Microcarpium* complex as discussed above, the relatively small amount of sequence data (only four chloroplast and one nuclear single-copy region), even though they provide a well-supported phylogeny, might not yet present a complete understanding of the evolutionary complexity of this

group. Additional single-copy nuclear genes and/or genome-wide data will be needed in further phylogenetic studies to test our evolutionary inference (e.g., Hipp et al., 2019; Valcárcel & Wen, 2019; White et al., 2019).

FURTHER IMPLICATIONS OF POLYPLOID AND RETICULATE EVOLUTION

Within the extensively polyploid *Microcarpium* complex, only five of the 20 extant species are diploids. Our analyses suggest that four or as many as eight diploid progenitors in the complex went extinct after they gave rise to the extant polyploids (Fig. 7). Although these diploid progenitors are extinct, their genomes are preserved in the extant polyploids, as detected by cytological work (Peng, 1988; Peng et al., 2005) and our molecular analysis (Fig. 7). Using molecular techniques, an increasing number of extinct diploid progenitors of extant polyploid plants have been detected in various groups, e.g., one or more extinct diploids in *Glycine* L. subg. *Glycine* (Doyle et al., 2004), two extinct diploids closely related to *Hordeum murinum* (Jakob & Blattner, 2010), and at least two extinct diploids in *Ludwigia* sect. *Macrocarpon* (Liu et al., 2018). The extinction of the diploid progenitors in the *Microcarpium* complex might be associated with climate cooling in the Tertiary, which drove extinctions in many other north-temperate plant lineages (Tiffney, 1985; Tiffney & Manchester, 2001; Wen et al., 2016). Moreover, many studies focusing on polyploids and their existing diploid progenitors have shown that polyploids often are more ecologically successful than related diploids, occupying larger geographical and ecological ranges (Levin, 1975; Lowry & Lester, 2006; te Beest et al., 2012). The ecologically more robust polyploid descendants may have outcompeted their diploid progenitors in the NTH *Ludwigia* clade, resulting in the extinction of many of the diploids in the lineage.

BIOGEOGRAPHIC INFERENCES

Analysis of geographic distribution (Fig. 6) supports earlier propositions that the evolution of the NTH *Ludwigia* clade centered in North America (Raven, 1963; Eyde, 1981; Tobe et al., 1988). The only exception is *L. ovalis*, which is endemic to East Asia, and *L. palustris*, which is widely distributed in North America, central and northern South America, Eurasia, and Africa (Raven, 1963; Peng et al., 2005; Wagner et al., 2007). Raven (1963) and Peng et al. (2005) proposed that *L. ovalis* and *L. palustris* may be relicts of the Arcto-Tertiary Geoflora. The report of Miocene fossil *Ludwigia* seeds from Denmark (Friis, 1985) that could be assigned to section *Isnardia* (Tobe et al., 1988) supports this proposition. Our *PgiC* tree indicates that the tetraploid *L. ovalis* is composed of genomes M_1 and M_2 (Figs. 5, 7), which are embedded within clades of the *Microcarpium* complex distributed in North America. Given this, *L. ovalis* must have originated in North America, dispersed to East Asia, possibly through the Bering land bridge (e.g., Li et al., 2015; Patiño et al., 2016; Wen et al., 2016), and subsequently become extinct in North America.

TAXONOMIC REVISION

Our analyses indicate that neither section *Isnardia* nor *Microcarpium* is monophyletic as currently circumscribed, and *Ludwigia ovalis* (monospecific section *Miquelia*) is an allotetraploid derived from two early-branching genomes in the *Microcarpium* complex (Figs. 4–6). By applying the principle of monophyly and using the earliest available name, we propose to combine the three sections into an expanded section *Isnardia* (L.) W. L. Wagner & Hoch (Wagner et al., 2007).

TAXONOMIC TREATMENT

Ludwigia L. sect. **Isnardia** (L.) W. L. Wagner & Hoch, Syst. Bot. Monogr. 83: 36. 2007. *Isnardia* L., Sp. Pl. 1: 120. 1753. TYPE: *Isnardia palustris* L. [≡ *Ludwigia palustris* (L.) Elliott].

Ludwigia sect. *Microcarpium* Munz, Bull. Torrey Bot. Club 71: 154. 1944, syn. nov. TYPE: *Ludwigia pilosa* Walter.

Ludwigia sect. *Miquelia* P. H. Raven, Reinwardtia 6: 337. 1963, syn. nov. TYPE: *Ludwigia ovalis* Miq.

Included species. *Ludwigia alata* Elliott, *L. arcuata* Walter, *L. brevipes* (Long) Eames, *L. curtissii* Chapm., *L. glandulosa* Walter subsp. *glandulosa*, *L. glandulosa* subsp. *brachycarpa* C.-I Peng, *L. lanceolata* Elliott, *L. linearis* Walter, *L. linifolia* Poir., *L. microcarpa* Michx., *L. ovalis* Miq., *L. palustris* (L.) Elliott, *L. pilosa* Walter, *L. polycarpa* Short & R. Peter, *L. ravenii* C.-I Peng, *L. repens* J. R. Forst., *L. simpsonii* Chapm., *L. spathulata* Torrey & A. Gray, *L. sphaerocarpa* Elliott, *L. stricta* (C. Wright ex Griseb.) C. Wright, *L. suffruticosa* Walter.

CONCLUSIONS

Using both the biparental nuclear single-copy *PgiC* region and four maternally inherited chloroplast regions (*rpL16*, *rpoB-trnC*, *trnL-trnF*, and *ycf6-psbM*), we reconstructed the phylogeny of the NTH *Ludwigia* group. These phylogenies indicate that the NTH *Ludwigia* clade is monophyletic, and that the diploid section *Ludwigia* and a redefined multiploid section *Isnardia* (*Microcarpium* complex, composed of former sections *Isnardia*, *Microcarpium*, and *Miquelia*) are

well-supported sister clades. Within the redefined section *Isnardia*, our results largely support hypotheses proposed in Peng (1988) and part of Peng et al. (2005) (Fig. 2). Eight genomes are recognized in the complex, and the reticulate evolution of each taxon in the section has been reconstructed (Fig. 7). Four, or possibly eight, extinct diploid progenitors that contributed to extant polyploids are also inferred in our phylogenies. The Tertiary climate change and the ecological success of the polyploids may have contributed to the extinction of the diploid progenitors.

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