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ORIGINAL ARTICLE

Two-way evolution of root form in the riverweed family Podostemaceae, with implications for phenotypic evolution

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Abstract
Whether phenotypic evolution is always adaptive is a major issue in evolutionary biology. Based on the great morphological variation and slight ecological variation, Willis argued that Podostemaceae evolved in the absence of adaptation. Podostemaceae are ecological specialists with roots or holdfasts adhering to macrophyte-free (in Asia), soilless rock surfaces submerged in fast-flowing river currents. In *Hydrobryum*, the roots are foliose and rarely ribbon-like. The ribbon-like rooted species are almost always sympatric with foliose-rooted species. The *mat*K phylogeny indicates that ribbon-like roots were derived recurrently from foliose roots. This late evolution is truly opposite to the early evolution, in which the foliose root of most *Hydrobryum* species was derived from the ribbon-like root of the common ancestor of the *Cladopus*–*Hydrobryum* clade. The two-way evolution and subsequent sympatry suggests that root forms do not necessarily affect the microdistribution of the species.

Keywords Adaptation · Epilithic root · Neutral evolution · Sympatry · Two-way evolution

Introduction

Plants are adapted to a wide range of environments over the earth and show specific adaptations to extreme environments, such as aquatic biospheres, high mountains, deserts, special soils, and so forth (Baker et al. 1992; Hoffman and Parsons 1997; Warton 2002; Körner 2003; Nagy and Grabherr 2009; Ramawat 2010; Evert and Eichhorn 2013). Among such plants, Podostemaceae are extreme aquatic haptophytes growing on waterworn rock surfaces in fast turbulent currents in rapids and waterfalls (Willis 1914; Sculthope 1967; Kato 2013). The currents sweeping the rock surfaces exclude macrophytes. Furthermore, Podostemaceae are restricted to seasonal fluctuations of water level in which the plants are exposed and flower above the lowered water level in the dry season. Such habitats are also inhabited by Hydrostachyaceae in some localities in Madagascar and Africa (Cusset 1972, 1973). Because Hydrostachyaceae do not occur outside those regions, they are not competitors of Podostemaceae, such as *Hydrobryum*, in Asia.

Although all habitats are in running water above stable rocks or solid substrates, the morphology of Podostemaceae is unique and diverse. In sharp contrast to the anchoring, absorbent subterranean roots of terrestrial plants, their root is a major adhering organ, while the stem, leaves and flowers are adventitious from the roots and, in Asian species of the subfamily Podostemoideae, the stem is usually reduced. Even in the early life history during early rainy season, the root, together with adhesive hairs on the hypocotyl, adheres to the rock surface. Some Podostemaceae do not have even a set of fundamental organs. A few genera are rootless (Imaichi et al. 2004; Cook and Rutishauser 2007; Koi and Kato 2010), and the stems of many genera are atypical, lacking a shoot apical meristem (Imaichi et al. 2005; Koi et al. 2005). Unlike most angiosperms and the Podostemaceae subfamilies Tristichoideae and Weddellinoideae, members of the Podostemoideae show borderless or fuzzy shoot-leaf

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morphology (Rutishauser 1995), which is consistent with the unique pattern of gene expression (Katayama et al. 2010).

The epilithic roots are flattened to various degrees and dorsiventral, playing the role of adhesion to rock surfaces, adaptation to fast currents and photosynthesis (like leaves), which are essential for the haptophytic life. The root form ranges from subcylindrical and ribbon-like to foliose or disk-like (Koi et al. 2006; Cook and Rutishauser 2007). In Asian Podostemoideae, the ribbon-like roots are characterized by their constant width, a single strand of elongate nontracheary cells running into the root apex and a root-borne shoot occurring at a sinus of root branches, whereas the foliose roots are characterized by their spreading width (fan shape), a network of strands of elongate nontracheary cells and root-borne shoots scattered on the dorsal surface of the root independently of root branching (Ota et al. 2001; Hiyama et al. 2002; Koi and Kato 2003; Koi et al. 2006). There are foliose roots (Fig. 1a) in, e.g., *Hanseniella*, *Hydrobryum*, *Thawatchaia* and *Zeylanidium* in Asia (Hiyama et al. 2002; Kato 2004; Koi and Kato 2012), while ribbon-like roots (Fig. 1b) are in, e.g., *Cladopus*, *Paracladopus* and *Zeylanidium* (Hiyama et al. 2002; Koi and Kato 2003, 2012; Kato 2006; Koi et al. 2008; Kato and Koi 2018). The root forms are produced by the root meristem, which is also dorsiventral to varying degrees (Koi et al. 2006). Koi et al. (2006) found that the root is primitive in the subfamilies Tristichoideae and Weddellinoideae, while the root of the derived Podostemoideae is specialized, being foliose with marginal meristems (Ota et al. 2001).

The aquatic Podostemaceae are sister to the phanerophytic Hypericaceae (Ruhfel et al. 2011; Xi et al. 2012). After the earliest adaptation during this drastic habitat change, the subsequent evolution and adaptation of root forms and other characters always took place in riverine habitats, where all species (> 300 spp.) evolved. It may be possible that diversification occurred in competitor-free habitats with relaxed natural selection. Based on his worldwide knowledge of the ecology, morphology and distribution, Willis (1914) argued that the great variation in Podostemaceae was produced in the absence of adaptation. This idea was taken over by van Steenis (1969, 1977, 1978, 1981). However, such evolution has not been demonstrated based on specific data.

In a phylogenetic analysis of the root form, Koi et al. (2006) and Moline et al. (2007) stressed that the foliose roots of *Hydrobryum* and a few allied genera are derived from the ribbon-like roots of, e.g., *Cladopus*. *Hydrobryum* is a most specialized genus of Asian Podostemoideae. Several species of *Hydrobryum* examined consist of a hypocotyl and two cotyledons but are devoid of a primary shoot and root in the embryo-seedling, and the root is adventitious and exogenous, instead of endogenous as in primitive genera and most other families (Suzuki et al. 2002; Koi et al. 2012b). The roots of 29 of the 33 species of *Hydrobryum*, as in allied genera, are foliose with leaves and shoots scattered irregularly on the dorsal surface (Fig. 1a) (Ota et al. 2001; Koi and Kato 2012, 2015b, 2018, 2019; Kato 2013, 2018). The roots of the remaining four species are ribbon-like with tufted leaves and flowers borne regularly in the sinuses of root branches (Fig. 1b) (Koi and Kato 2012, 2018), quite similar to the roots of *Cladopus*. Although molecular phylogenetic data indicate that the ribbon-like species of *Hydrobryum* are related to foliose species, their evolutionary processes have not been discussed (Koi and Kato 2012, 2019). In spite of the different root forms, the species are sympatric. However, distribution information is incomplete and requires further field data to determine the microdistribution.

The aim of this study was to examine whether the evolution of the root form in *Hydrobryum* is adaptive, by

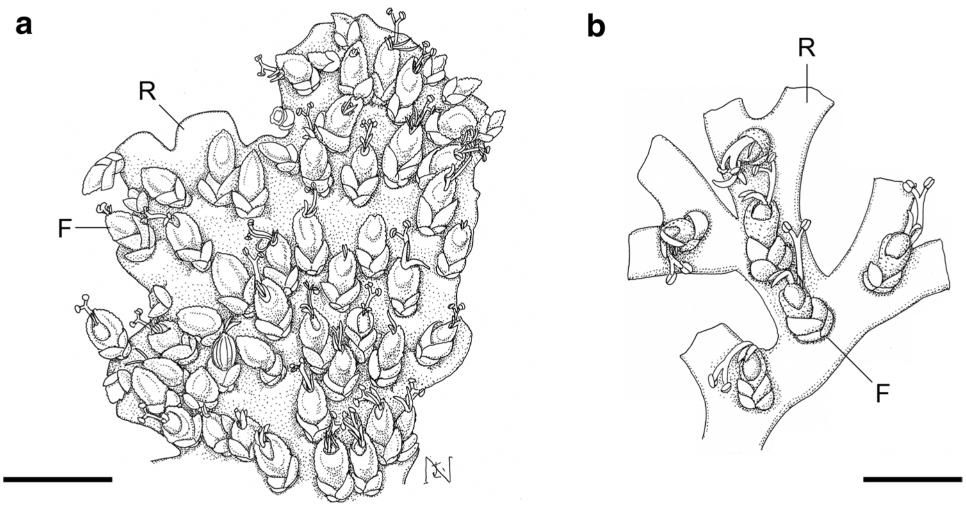


Fig. 1 Morphology of Podostemaceae roots. **a** Foliose root (*Hydrobryum subcrustaceum*). **b** Ribbon-like root (*H. taeniatum*). *F* flower; *R* root. Scale bars = 5 mm (**a**) and 3 mm (**b**). The illustrations are reproduced from Koi and Kato (2012)

Table 1 Materials used in morphological observations

Species	Source and voucher
Hydrobryum clandestinum	Laos. Bolikhamxay Prov.: waterfall in Xai stream (main stream flowing into Tad Xai), upstream of Tad Xai, Phou Khao Khouay National Protected Area (= PKK), 18° 28′ 01.1″ N, 103° 07′ 41.2″ E, *Koi* et al. *LPK-205* (Loc. 6)
	Laos. Bolikhamxay Prov.: Tad Heua Hak waterfall, Ban Xaisavang, Pakxan District, PKK, 18° 28′ 23.8″ N, 103° 31′ 20.2″ E, *Koi* et al. *LPK-03* (Loc. 9)
Hydrobryum hapteron	Laos. Bolikhamxay Prov.: rapids in tributary upstream of Tad Xai, PKK, 18° 28′ 15.5″ N, 103° 07′ 23.0″ E, *Kato L-513*, *Koi* et al. *LPK-06* (Loc. 7)
	Laos. Bolikhamxay Prov.: rapids in Xai stream (main stream flowing into Tad Xai), upstream of Tad Xai, PKK, 18° 28′ 15.5″ N, 103° 07′ 22.9″ E, *Koi* et al. *LPK-208* (Loc. 7)
Hydrobryum ramosum	Laos. Vientiane Prov.: Tad Omong near Nam Mang 3 Dam, Ban Vanghuea, PKK, 18° 21′ 38.0″ N, 102° 48′ 26.3″ E, *Kato L-505*, *Koi* et al. *LPK-20* (Loc. 2)
Hydrobryum stellatum	Laos. Bolikhamxay Prov.: Tad Yong, PKK, 18° 21′ 45.8″ N, 103° 04′ 50.4″ E, *Koi* et al. *LPK-10*, *Koi* et al. *Kato-11-22-4* (Loc. 3)
Hydrobryum subcrustaceum	Laos. Vientiane Prov.: Chaova waterfall, PKK, 18° 22′ 07.9″ N, 102° 42′ 13.3″ E, *Kato* et al. *L-15* (Loc. 1)
	Laos. Vientiane Prov.: Nam Mang 3 Dam, PKK, 18° 21′ 35.9″ N, 102° 48′ 25.3″ E, *Koi & Wongprasert LK-03*, *Koi* et al. *LKF-02*, *Koi* et al. *LKF-04* (Loc. 2)
Hydrobryum subcylindricoides	Laos. Bolikhamxay Prov.: rapids in tributary upstream of Tad Xai, PKK, 18° 28′ 15.5″ N, 103° 07′ 23.0″ E, *Kato L-513*, *Koi* et al. *LPK-07* (Loc. 7)
Hydrobryum subcylindricum	Laos. Bolikhamxay Prov.: Tad Yong, PKK, 18° 21′ 45.8″ N, 103° 04′ 50.4″ E, *Koi* et al. *LPK-12*, *Koi* et al. *LPK-13B*, *Koi* et al. *Kato-11-22-3*, *Koi* et al. *Kato-11-22-2*, *Koi* et al. *Kato-11-22-5* (Loc. 3)
	Laos. Bolikhamxay Prov.: waterfall in Yong River, upstream of Tad Yong waterfall, PKK, 18° 22′ 03.9″ N, 103° 04′ 03.9″ E, *Koi* et al. *LPK-210* (Loc. 3-1)
	Laos. Bolikhamxay Prov.: Tad Leuk waterfall, PKK, 18° 23′ 42.9″ N, 103° 04′ 17.0″ E, *Kato* et al. *L-09* (Loc. 4)
	Laos. Bolikhamxay Prov.: Tad Xai waterfall, PKK, 18° 27′ 07.0″ N, 103° 08′ 39.5″ E, *Kato* et al. *L-13*, *Koi* et al. *LKF-17* (Loc. 5)
Hydrobryum taeniatum	Laos. Bolikhamxay Prov.: Tad Yong, PKK, 18° 21′ 45.8″ N, 103° 04′ 54.0″ E, *Koi* et al. *LPK-13A*, *Koi* et al. *LPK-14* (Loc. 3)
	Laos. Bolikhamxay Prov.: waterfall in Yong river, upstream of Tad Yong, PKK, 18° 22′ 03.9″ N, 103° 04′ 03.9″ E, *Koi* et al. *LPK-211* (Loc. 3-1)
	Laos. Bolikhamxay Prov.: tributary of Yong river, upstream of Tad Yong, PKK, 18° 21′ 49.4″ N, 103° 03′ 50.7″ E, *Koi* et al. *LPK-209* (Loc. 3-2)
	Laos. Bolikhamxay Prov.: Tad Leuk waterfall, PKK, 18° 23′ 42.9″ N, 103° 04′ 17.0″ E, *Kato* et al. *L-08* (Loc. 4)
	Laos. Bolikhamxay Prov.: Rapids 1 km upstream from Tad Leuk, PKK, 18° 23′ 58.0″ N, 103° 03′ 54.4″ E, *Koi* et al. *LPK-19* (Loc. 4-1)
	Laos. Bolikhamxay Prov.: Tad Lo, Nam Lo river, Ban Si Som Xay, PKK, 18° 27′ 02.9″ N, 103° 17′ 48.7″ E, *Koi* et al. *LPK-215*, *Koi* et al. *LPK-216* (Loc. 8)
	Laos. Bolikhamxay Prov.: Tad Nampa waterfall, Nam Nampa R., Ban Nampa, 18° 30′ 47.7″ N, 103° 36′ 26.8″ E, *Koi & Wongprasert LK-107* (Loc. 10)
Hydrobryum takakioides	Laos. Bolikhamxay Prov.: Tad Xai waterfall, PKK, 18° 27′ 18.8″ N, 103° 08′ 31.8″ E, *Kato* et al. *L-14*, *Koi & Wongprasert LK-202*, *Koi* et al. *LKF-16* (Loc. 5)
Hydrobryum verrucosum	Laos. Bolikhamxay Prov.: Tad Yong, 18° 21′ 45.8″ N, 103° 04′ 50.6″ E, *Koi* et al. *LPK-11*, *Koi* et al. *Kato-11-22-7*, *Koi* et al. *Kato-11-22-1* (Loc. 3)
	Laos. Bolikhamxay Prov.: waterfall in Yong River, upstream of Tad Yong, PKK, 18° 22′ 03.9″ N, 103° 04′ 03.9″ E, *Koi* et al. *LPK-212* (Loc. 3-1)
	Laos. Bolikhamxay Prov.: Tad Leuk waterfall, PKK, 18° 23′ 42.9″ N, 103° 04′ 17.0″ E, *Kato* et al. *L-07*; rapids 1 km upstream, 18° 23′ 58.0″ N, 103° 03′ 54.4″ E, *Koi* et al. *LPK-17* (Loc. 4)
	Laos. Bolikhamxay Prov.: Tad Xai waterfall, PKK, 18° 27′ 07.0″ N, 103° 08′ 39.5″ E, *Kato* et al. *L-12*, *Koi* et al. *LKF-14* (Loc. 5)
	Laos. Bolikhamxay Prov.: waterfall of Xai stream (main stream flowing into Tad Xai), upstream of Tad Xai, PKK, 18° 28′ 01.1″ N, 103° 07′ 41.2″ E, *Koi* et al. *LPK-204* (Loc. 6)
	Laos. Bolikhamxay Prov.: Tad Nampa waterfall, Nam Nampa R., Ban Nampa, 18° 30′ 47.7″ N, 103° 36′ 26.8″ E, *Koi & Wongprasert LK-105* (Loc. 10)
	Laos. Khammouan Prov.: hills NW of Ban Mak Pheuang on N side of Nam Thun, 17° 51′ 47″ N, 105° 20′ 2″ E, *Newman* et al. *LAO 708* (E)

analyzing its phylogeny and comparing it with the microdistribution of the species.

Materials and methods

Materials and collection sites

Hydrobryum hapteron, *H. ramosum*, *H. stellatum*, *H. subcrustaceum*, *H. subcylindricoides*, *H. subcylindricum*, *H. taeniatum*, *H. takakioides* and/or *H. verrucosum* occur in ten waterfalls and rapids in one mountain range northeast of Vientiane, Laos. Six waterfalls and rapids flow into one tributary of the Mekong River. Most sites are in the Phou Khao Khouay National Protected Area (NPA), and the Tad Nampa waterfall is near the eastern margin of the Area. Materials collected were dried with silica gel for molecular phylogenetic analysis (Online Resource 1) or fixed with FAA (formalin/acetic acid/50% ethyl alcohol = 5:5:90 in volume) for morphological observations (Table 1). Vouchers are deposited in the Department of Botany, National Museum of Nature and Science (TNS) and the National Herbarium of Laos (HNL).

The localities of the materials are generally from east to west. The easternmost and westernmost waterfalls are nearly 100 km apart, while Tad Leuk and the rapids upstream of Tad Xai are about 3 km apart from Tad Yong and Tad Xai, respectively (Fig. 2). Each waterfall is inhabited by two or more, rarely one species of *Hydrobryum*. Species of *Dalzellia*, *Hydrodiscus* and *Polypleurum* also coexist with the species of *Hydrobryum* (Koi and Kato 2012, 2015a, b). In other waterfalls within the NPA, there is *Hydrobryum vientianense*, which belongs to another clade (Koi and Kato 2012). Species that are not monophyletic with *H. austrolaoticum* (see Results) were excluded from analysis. The species of *Hydrobryum* analyzed, except *H. verrucosum*, were not found outside the Area during 13 recent explorations in Laos (Koi and Kato 2012, 2015a, b, 2018, 2019; Koi et al. 2019) (Table 1).

Fig. 3 Phylogenetic relationships of **a** *Hydrobryum* and allies (i.e., *Cladopus–Hydrobryum* clade), and **b** *Zeylanidium* and allies (i.e., *Polypleurum–Zeylanidium* clade) inferred from RAxML analysis based on *mat*K sequence. Monophyletic species of podostemoid genera related to *Hydrobryum* are represented by triangles (with vertical width reflecting the number of samples). Support values (≥ 50) of RAxML and MP are shown above and below branches. Asterisks indicate branches supported with 100% bootstrap values of RAxML and MP analyses ▶

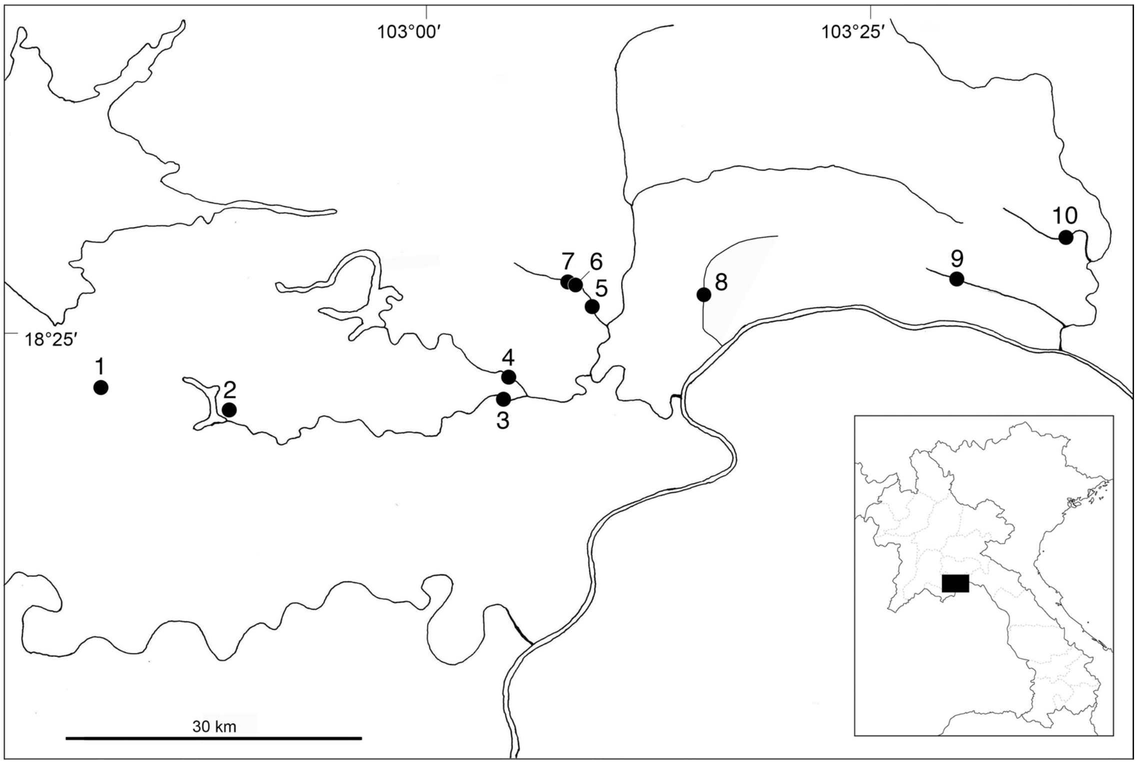
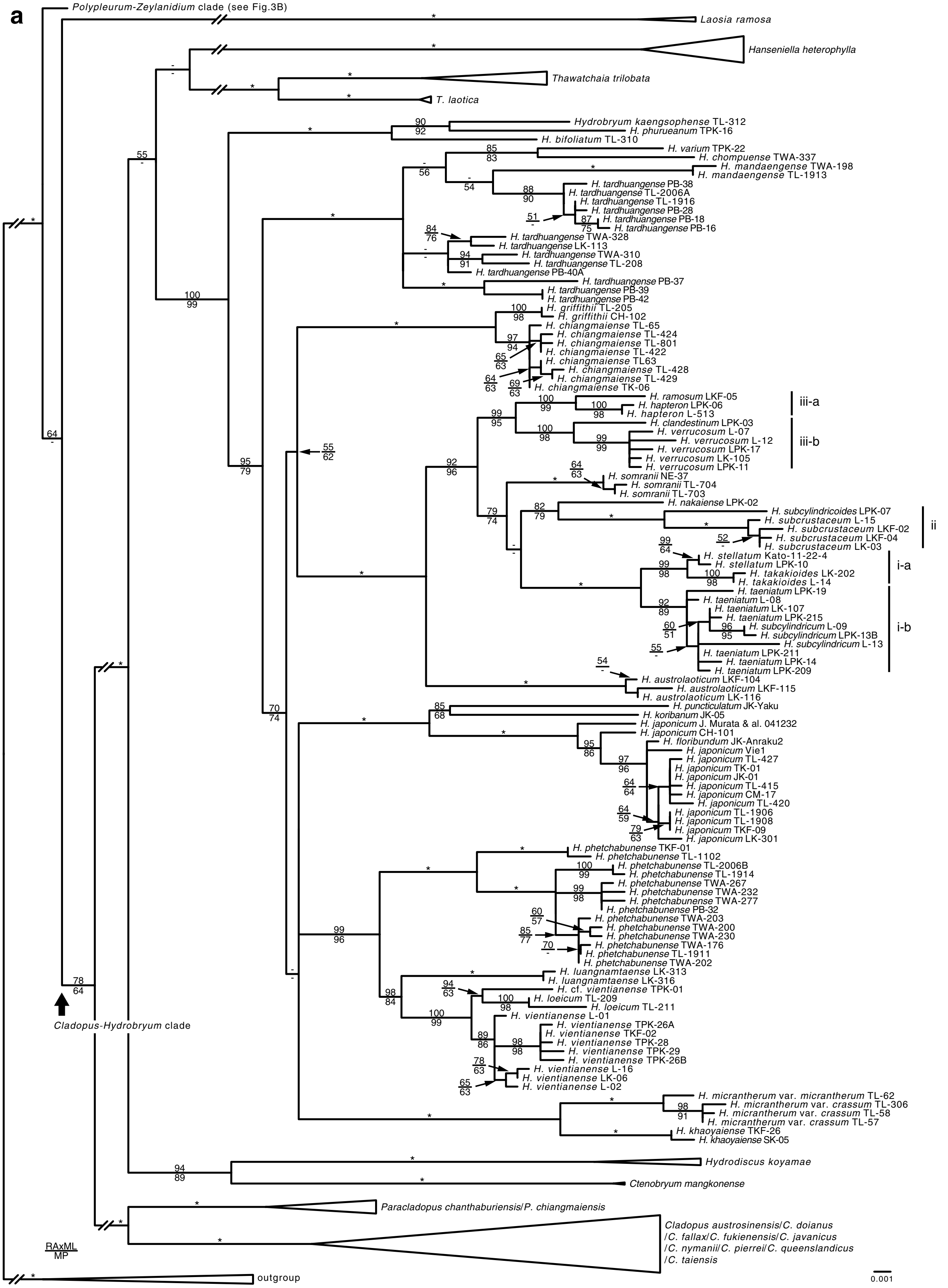


Fig. 2 Map showing localities (rapids or waterfalls) of species of *Hydrobryum* in Phou Khao Khouay NPA. Inset shows location in Laos. 1, Chaova; 2, Nam Mang 3 Dam; 3, Tad Yong; 4, Tad Leuk; 5, Tad Xai; 6, waterfall in Xai stream, upstream of Tad Xai; 7, rapids upstream of Tad Xai; 8, Tad Lo; 9, Tad Heua Hak; and 10, Tad Nampa. The numbers correspond with those in Table 2


a
Polypleurum-Zeylanidium clade (see Fig.3B)
Laosia ramosa
Hanseniella heterophylla
Thawatchaia trilobata
T. laotica
Hydrobryum kaengsophense TL-312
H. phurueanum TPK-16
H. bifoliatum TL-310
H. varium TPK-22
H. chompuense TWA-337
H. mandaengense TWA-198
H. mandaengense TL-1913
H. tardhuangense PB-38
H. tardhuangense TL-2006A
H. tardhuangense TL-1916
H. tardhuangense PB-28
H. tardhuangense PB-18
H. tardhuangense PB-16
H. tardhuangense TWA-328
H. tardhuangense LK-113
H. tardhuangense TWA-310
H. tardhuangense TL-208
H. tardhuangense PB-40A
H. tardhuangense PB-37
H. tardhuangense PB-39
H. tardhuangense PB-42
H. griffithii TL-205
H. griffithii CH-102
H. chiangmaiense TL-65
H. chiangmaiense TL-424
H. chiangmaiense TL-801
H. chiangmaiense TL-422
H. chiangmaiense TL63
H. chiangmaiense TL-428
H. chiangmaiense TL-429
H. chiangmaiense TK-06
H. ramosum LKF-05
H. hapteron LPK-06
H. hapteron L-513
iii-a
H. clandestinum LPK-03
H. verrucosum L-07
H. verrucosum L-12
H. verrucosum LPK-17
H. verrucosum LK-105
H. verrucosum LPK-11
iii-b
H. somranii NE-37
H. somranii TL-704
H. somranii TL-703
H. nakaiense LPK-02
H. subcylindricoides LPK-07
H. subcrustaceum L-15
H. subcrustaceum LKF-02
H. subcrustaceum LKF-04
H. subcrustaceum LK-03
ii
H. stellatum Kato-11-22-4
H. stellatum LPK-10
H. takakioides LK-202
H. takakioides L-14
i-a
H. taeniatum LPK-19
H. taeniatum L-08
H. taeniatum LK-107
H. taeniatum LPK-215
H. subcylindricum L-09
H. subcylindricum LPK-13B
H. subcylindricum L-13
H. taeniatum LPK-211
H. taeniatum LPK-14
H. taeniatum LPK-209
i-b
H. austrolaoticum LKF-104
H. austrolaoticum LKF-115
H. austrolaoticum LK-116
H. puncticulatum JK-Yaku
H. koribanum JK-05
H. japonicum J. Murata & al. 041232
H. japonicum CH-101
H. floribundum JK-Anraku2
H. japonicum Vie1
H. japonicum TL-427
H. japonicum TK-01
H. japonicum JK-01
H. japonicum TL-415
H. japonicum CM-17
H. japonicum TL-420
H. japonicum TL-1906
H. japonicum TL-1908
H. japonicum TKF-09
H. japonicum LK-301
H. phetchabunense TKF-01
H. phetchabunense TL-1102
H. phetchabunense TL-2006B
H. phetchabunense TL-1914
H. phetchabunense TWA-267
H. phetchabunense TWA-232
H. phetchabunense TWA-277
H. phetchabunense PB-32
H. phetchabunense TWA-203
H. phetchabunense TWA-200
H. phetchabunense TWA-230
H. phetchabunense TWA-176
H. phetchabunense TL-1911
H. phetchabunense TWA-202
H. luangnamtaense LK-313
H. luangnamtaense LK-316
H. cf. vientianense TPK-01
H. loeicum TL-209
H. loeicum TL-211
H. vientianense L-01
H. vientianense TPK-26A
H. vientianense TKF-02
H. vientianense TPK-28
H. vientianense TPK-29
H. vientianense TPK-26B
H. vientianense L-16
H. vientianense LK-06
H. vientianense L-02
H. micrantherum var. micrantherum TL-62
H. micrantherum var. crassum TL-306
H. micrantherum var. crassum TL-58
H. micrantherum var. crassum TL-57
H. khaoyaiense TKF-26
H. khaoyaiense SK-05
Hydrodiscus koyamae
Ctenobryum mangkonense
Paracladopus chanthaburiensis/P. chiangmaiensis
Cladopus austrosinensis/C. doianus
/C. fallax/C. fukienensis/C. javanicus
/C. nymanii/C. pierrei/C. queenslandicus
/C. taiensis
Cladopus-Hydrobryum clade
RAxML
MP
outgroup
0.001

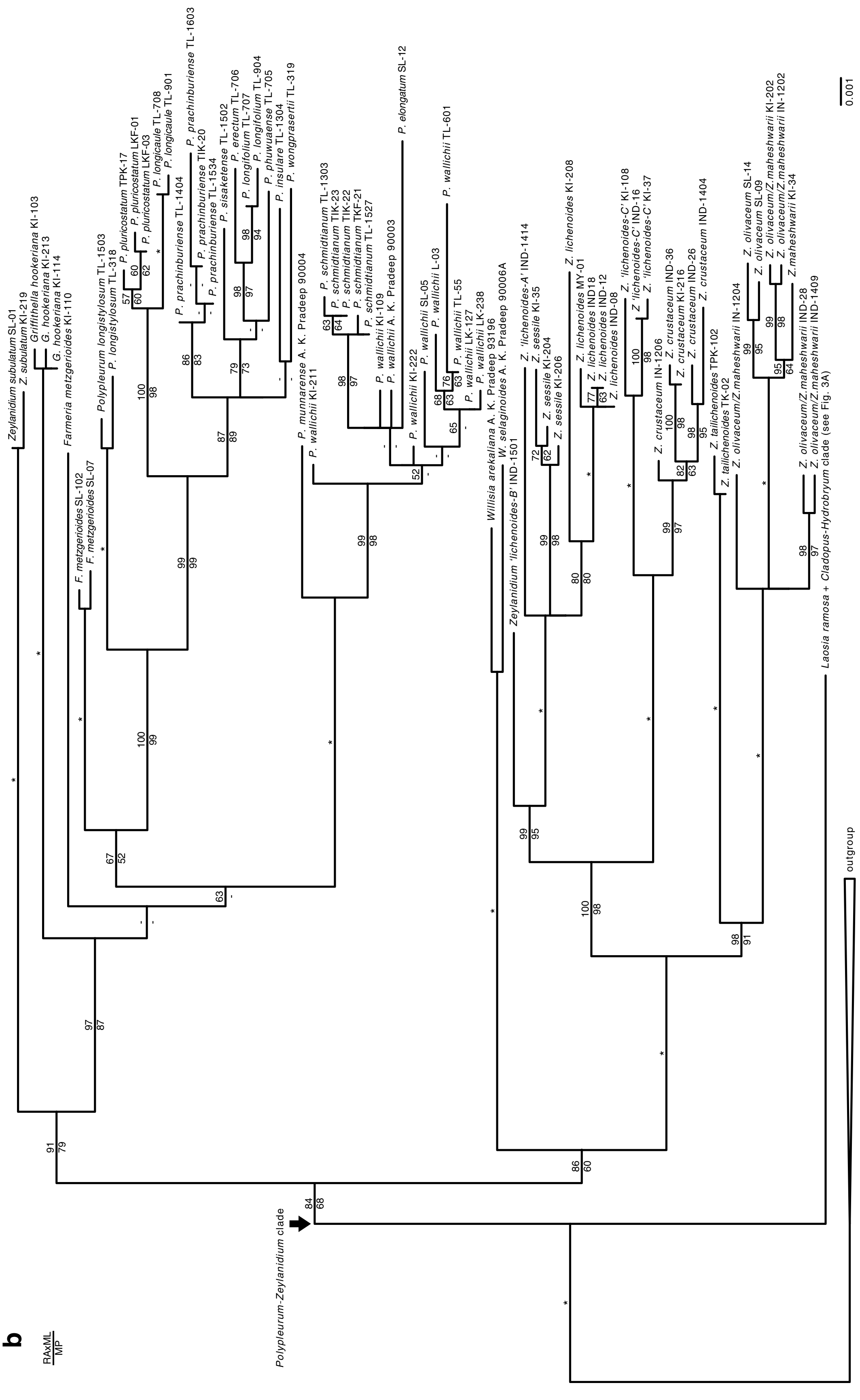


Fig. 3 (continued)

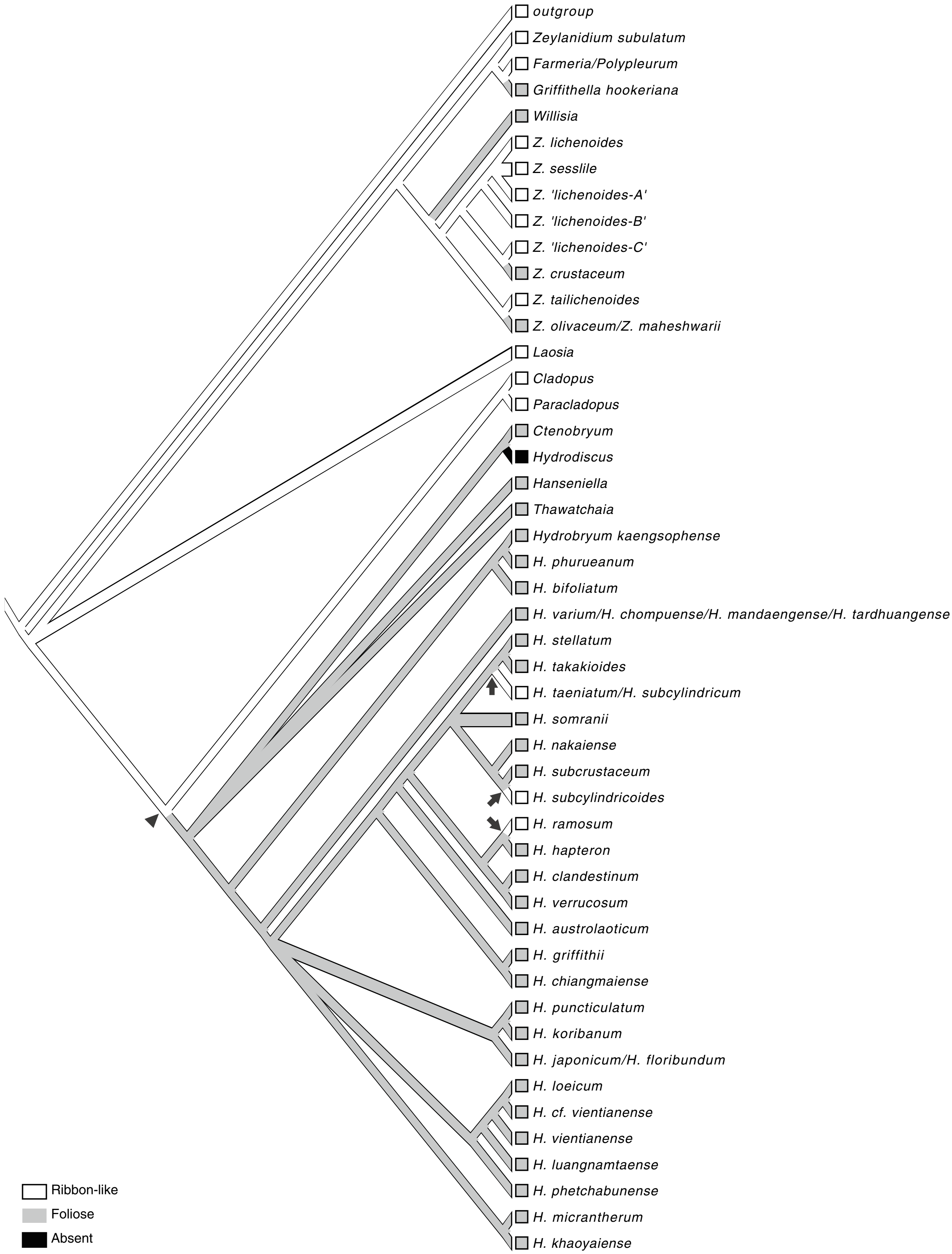
Fig. 4 Roots of *Hydrobryum* species. **a** Foliose root of *Hydrobryum clandestinum* with leaves scattered on dorsal surface. **b** Ribbon-like root of *H. taeniatum* with leaves in sinuses of root branches. Note shoot primordium in green color appeared at root apex. **c–h** Foliose (**c**, **e**, **g**) and ribbon-like (**d**, **f**, **h**) roots of sister species of *Hydrobryum*. **c** *Hydrobryum hapteron*, **d** *H. ramosum*, **e** *H. stellatum*, **f** *H. subcylindricum*, **g** *H. subcrustaceum*, **h** *H. subcylindricoides*. Asterisks indicate root branches. Arrows indicate tufted leaves (**a**, **b**), flower buds (**c**, **d**, **e**, **h**) or fruits (**f**, **g**). Scale bars = 5 mm

Molecular phylogeny

Chloroplast *mat*K gene sequences (1524 bp) were determined using the methods described previously by Koi and Kato (2010). Sequences deposited in GenBank were used for phylogenetic analyses (Online Resource 1). The sequences were aligned by Clustal X ver. 2.1 (Larkin et al. 2007) and refined manually with Mesquite ver. 3.40 (Maddison and Maddison 2017) (Online Resource 2). Gaps were treated as missing data. The program MrModeltest 2.3 (Nylander 2004) determined a general time reversible (GTR) + proportion of invariable sites (I) + shape parameter of the gamma distribution (G) substitution model as the best fitting model of substitution: Nucleotide frequencies were $A = 0.3342$, $C = 0.1385$, $G = 0.1231$, $T = 0.4042$; the substitution rate matrix was A to $C = 1.1948$, A to $G = 1.0928$, A to $T = 0.2083$, C to $G = 0.4480$, C to $T = 0.8737$, G to $T = 1.0000$; and the proportion of invariable sites was 0.2425. The gamma distribution shape parameter was 0.8650. Maximum likelihood (ML) analysis was conducted using RAxML-HPC2 (Stamatakis 2014) on XSEDE (8.2.10) in Cipres Science Gateway (Miller et al. 2010) with GTR + I + G model. Bootstrap probability (BP) values were calculated for 1000 replicates. In maximum parsimony (MP) analyses with the program PAUP* Version 4.0a159 (Swofford 2002), all characters were equally weighted, and bootstrap values were calculated for 10,000 replicates with ten random addition replicates involving tree-bisection-reconnection (TBR) branch swapping; the 'MulTrees' option was not in effect. *Endocaulos mangorense* and *Thelethylax minutiflora* were used as outgroups (Koi et al. 2012a).

Morphology

For morphological studies, we used specimens preserved in FAA. The roots were observed under a stereo microscope (Leica M125, Heerbrugg, Switzerland), focusing on the site where shoots are formed. Observations considered the


outgroup
Zeylanidium subulatum
Farmeria/Polypleurum
Griffithella hookeriana
Willisia
Z. lichenoides
Z. sesslile
Z. 'lichenoides-A'
Z. 'lichenoides-B'
Z. 'lichenoides-C'
Z. crustaceum
Z. tailichenoides
Z. olivaceum/Z. maheshwarii
Laosia
Cladopus
Paracladopus
Ctenobryum
Hydrodiscus
Hanseniella
Thawatchaia
Hydrobryum kaengsophense
H. phurueanum
H. bifoliatum
H. varium/H. chompuense/H. mandaengense/H. tardhuangense
H. stellatum
H. takakioides
H. taeniatum/H. subcylindricum
H. somranii
H. nakaiense
H. subcrustaceum
H. subcylindricoides
H. ramosum
H. hapteron
H. clandestinum
H. verrucosum
H. australaoticum
H. griffithii
H. chiangmaiense
H. puncticulatum
H. koribanum
H. japonicum/H. floribundum
H. loeicum
H. cf. vientianense
H. vientianense
H. luangnamtaense
H. phetchabunense
H. micrantherum
H. khaoyaiense
Ribbon-like
Foliose
Absent

◂**Fig. 5** Phylogenetic relationship of root form in Asian Podostemoideae. Arrowhead indicates the common ancestor of the *Cladopus–Hydrobryum* clade. Arrows indicate the branching points where root form evolved from foliose to ribbon-like

following developmental features of the roots and shoots: In *Cladopus* allied to *Hydrobryum*, the shoots are initiated within the root meristem and the root is divided into two unequal branches with shoots borne in the sinuses of the root branches (Koi and Kato 2003). A quite similar mode of shoot development is reported for *Zeylanidium*, which is phylogenetically far from the *Cladopus–Hydrobryum* clade (Hiyama et al. 2002). In comparison, in the foliose roots of *Hydrobryum* and *Zeylanidium*, the shoots are formed proximally to the root meristem and are not involved in the division of the root, so that the shoots are scattered on the surface of the root (Fig. 1a; Ota et al. 2001; Hiyama et al. 2002).

Character phylogeny

The evolution of the root form was reconstructed onto the present phylogenetic tree of the species using the program Mesquite ver. 3.31 (Maddison and Maddison 2017). The character states, foliose and ribbon-like, were derived from our previous studies (Koi and Kato 2012, 2015b, 2018, 2019; Kato 2013, 2018; Koi et al. 2019).

Results

Phylogeny

Hydrobryum was divided into three clades, i.e., a clade of *H. bifoliatum*/*H. kaengsophense*/*H. phurueanum*, a clade of *H. chompuense*/*H. mandaegense*/*H. tardhuangense*/*H. varium* and the remaining species (Fig. 3a). The former two clades were supported with 100% bootstrap values in both RAxML and MP analyses, while the latter was supported with 70% and 74% bootstrap values in RAxML and MP analyses, respectively. The last clade was subdivided into five subclades with robust support. One of the subclades was further divided into *H. austrolaoticum* and the rest. In the last subclade, the three species with ribbon-like roots were separately sister to the three species with foliose roots: The group (Fig. 3a, Clade i-b) of *H. taeniatum* and *H. subcylindricum* was sister to a group (Clade i-a) of *H. takakioides* and *H. stellatum* (Clade i); *Hydrobryum subcylindricoides* was sister to *H. subcrustaceum* (Clade ii); and the group (Clade iii-a) of *H. ramosum* and *H. hapteron* was sister to the group (Clade iii-b) of *H. verrucosum* and *H. clandestinum.*

Root morphology and character phylogeny

The roots of *H. clandestinum*, *H. hapteron*, *Hydrobryum stellatum* and *H. subcrustaceum*, as well as *H. takakioides* and *H. verrucosum* (data not shown), are foliose with irregular lobes (Figs. 1a, 4a, c, e, g). The vegetative shoots consisting of tufted leaves and the floriferous shoots are scattered on the dorsal surface of the roots with no or little relationship to the division of the roots. The roots of *H. taeniatum*, *H. ramosum*, *H. subcylindricum* and *H. subcylindricoides* are ribbon-like (Figs. 1b, 4b, d, f, h). They are sympodially or dichotomously branched with the vegetative shoots or floriferous shoots in the sinuses of root branches. This morphology is quite similar to that of *Cladopus* and other related genera in Asian podostemoids. We observed young shoots in the root meristematic areas in *H. taeniatum* (Fig. 4b) and *H. ramosum* (data not shown), like in *Zeylanidium lichenoides* (Hiyama et al. 2002), although histological data are not available for shoot development.

The character phylogeny of root form showed that the foliose root is an ancestral state in *Hydrobryum*, while the ribbon-like root is a derived state (Fig. 5). The ribbon-like roots were derived three times from the foliose roots in the clade of *H. stellatum*, *H. takakioides*, *H. taeniatum* and *H. subcylindricum*, in the clade of *H. subcrustaceum* and *H. subcylindricoides* and in the clade of *H. hapteron* and *H. ramosum* (Fig. 5, arrows). In contrast, the ribbon-like root is ancestral in the common ancestor of the *Cladopus* and *Hydrobryum* clades (Fig. 5, arrowhead). In the *Polypleurum–Zeylanidium* clade, the foliose root is derived recurrently in *Griffithella hookeriana*, *Willisia*, *Z. crustaceum* and *Z. olivaceum*/*Z. maheshwarii* (Figs. 3b, 5).

Distribution

In total, 15 species were found in ten waterfalls and rapids in the Phou Khao Khouay NPA (Table 2, Fig. 2). Eight of the localities (Loc. 2–8, 10) are inhabited by sympatric species. Five foliose or ribbon-like species, i.e., *H. taeniatum*, *H. subcylindricum*, *H. subcrustaceum*, *H. verrucosum* and *H. clandestinum*, each occur in two or more localities, while the other five, *H. takakioides*, *H. stellatum*, *H. subcylindricoides*, *H. hapteron* and *H. ramosum*, are restricted to single localities. The foliose and ribbon-like species are sympatric in six localities (Loc. 2–5, 7, 10) and occasionally occur even on the same rock.

Table 2 Microdistribution of sister species of *Hydrobryum* and species of other genera in waterfalls or rapids in Phou Khao Khouay NPA. Clades correspond with those in Fig. 3

Species	Clade	Loc. 1	Loc. 2	Loc. 3*	Loc. 4*	Loc. 5*	Loc. 6	Loc. 7	Loc. 8	Loc. 9	Loc. 10
H. takakioides	i-a					F					
H. stellatum	i-a			F							
H. taeniatum	i-b			R	R				R		R
H. subcylindricum	i-b			R	R	R					
H. subcrustaceum	ii	F	F								
H. subcylindricoides	ii							R			
H. hapteron	iii-a							F			
H. ramosum	iii-a		R								
H. verrucosum	iii-b			F	F	F	F				F
H. clandestinum	iii-b						F			F	
Dalzellia pseudoangustissima	n.a.			X	X	X			X		
Hydrodiscus koyamae	n.a.		X	X	X	X					X
Laosia ramosa	n.a.							X			
Polypleurum pluricostatum	n.a.		X								
Polypleurum schmidtianum	n.a.										X

Loc. 1 Chaova; *Loc. 2* Nam Mang 3 Dam; *Loc. 3* Tad Yong (including nearby tributary and waterfall); *Loc. 4* Tad Leuk (including nearby rapids); *Loc. 5* Tad Xai; *Loc. 6* waterfall in Xai stream, upstream of Tad Xai; *Loc. 7* rapids upstream of Tad Xai; *Loc. 8* Tad Lo; *Loc. 9* Tad Heua Hak; and *Loc. 10* Tad Nampa. Numbers of Localities correspond with those in Fig. 2

F foliose root, *R* ribbon-like root, *X* indicates the presence of non-*Hydrobryum* species

Asterisks (*) indicate localities where foliose-rooted species and ribbon-like-rooted ones occur on the same rocks

Discussion

Two-way evolution

The study of character evolution suggests that the foliose root recently changed three times to the ribbon-like root in *Hydrobryum*. In comparison, the foliose roots had earlier been derived from the ribbon-like roots of the common ancestor of *Cladopus*, *Paracladopus*, *Ctenobryum*, *Hydrodiscus*, *Hanseniella*, *Thawatchaia* and *Hydrobryum* and was derived recurrently in *Griffithella hookeriana*, *Willisia* (2 spp.) and *Zeylanidium* pro parte (present study; Koi et al. 2006; Moline et al. 2007). The ribbon-like roots of *Hydrobryum* exactly share the root–shoot spatial relationship with those of *Cladopus*, *Zeylanidium* and several species of *Polypleurum* (Mathew and Satheesh 1997; Kato 2006). Taking into account the specific mode of root–shoot development, as noted in Introduction and Methods (Ota et al. 2001; Hiyama et al. 2002; Koi and Kato 2003), and the recurrent sister–species relationships of root forms, the derived and the ancestral ribbon-like roots are comparable with each other. Therefore, we estimate that evolution of the root form is two-way and recurrent in the *Cladopus*–*Hydrobryum* clade. It is plausible that the derived ribbon-like root is produced by a slight genetical change in the process of development, e.g., a reuse of the pathway of the ancestral root, although comparative developmental and molecular genetic studies are necessary to reveal the evolutionary mechanism.

Sympatry

The foliose species (F) and ribbon-like species (R) of *Hydrobryum* are sympatric in six of ten localities in the area (Loc. 2–5, 7, 10 in Table 2). The two types of roots are semicircular in outline because root branching occurs near the apex of the ribbon roots, and the species of the two types usually occur on different rocks or separately on the same wide rocks. Among them, two species in Nam Mang 3 Dam (Loc. 2) are sister to those in rapids upstream of Tad Xai (Loc. 7): *H. subcrustaceum* (F) to *H. subcylindricoides* (R) (Clade ii) and *H. ramosum* (R) to *H. hapteron* (F) (Clade iii-a). Most likely, they are products of allopatric speciation. [Allopatric speciation is also suggested for *H. takakioides* (F) and *H. stellatum* (F) (Clade i-a), and less clearly for *H. verrucosum* (F) and *H. clandestinum* (F) (Clade iii-b), although the sister species retain the same root form.] These allopatric species are sympatric with species of different phylogenies. The sympatry in Loc. 2 and Loc. 7 perhaps appeared at the time of speciation. By contrast, the ribbon-like *H. taeniatum* and *H. subcylindricum* (Clade i-b) diverged from the common ancestor with foliose *H. takakioides* and *H. stellatum* (Clade i-a), indicating a longer history of sympatry in Loc. 3 and Loc. 5. Sympatry in the other Loc. 4 and Loc. 10 is shown by ribbon-like and foliose species with other patterns of phylogeny. Such frequent and recurrent sympatry strongly suggests that the two root forms have equivalent adhesion capability of roots, which is essential for the haptophytic life, although other differences are not excluded.

In other areas, too, foliose species of *Hydrobryum* are sympatric with ribbon-like species of *Cladopus* in, e.g., two waterfalls in Khao Yai National Park, central Thailand; a waterfall in Sa Kaeo, eastern Thailand; and two rapids in Kagoshima, Japan (Kato 2004, 2006, 2008; Koi and Kato 2012; Werukamkul et al. 2018; Kato unpubl. observations). Furthermore, species of other genera with different root forms or holdfasts coexist in the same localities worldwide (Table 2; Willis 1914; Philcox 1996; Mathew and Satheesh 1997; Novelo and Philbrick 1997; Rutishauser 1997; Tur 1997; Kato 2004, 2006; Lin et al. 2016; Werukamkul et al. 2012; Kato and Koi unpubl. observations). Sympatry in a wide range of species and areas strengthens the suggested equivalent capability of the different root forms.

Nonadaptive evolution

Contrary to neutral evolution at the molecular level (Kimura 1983), phenotypic evolution is generally accepted as being adaptive, but Lewontin (1978) using the number of horns of rhinoceros and other examples, Nei (1987, 2013), Stearns and Hoekstra (2005) and Saitou (2009) stressed that it may not always be so. Darwin (1859) also mentioned that natural selection has been the main but not the exclusive means of modification. From the viewpoint of adaptive evolution (e.g., Bell 2008; Futuyma 2009), theoretically, in the early evolution, the derived foliose root of the *Hydrobryum* clade would have gained larger advantages than the ancestral ribbon-like root. Given that this trend continued in the late evolution, as seen in the *Polypleurum–Zeylanidium* clade (this study), the derived ribbon-like root in *Hydrobryum* should have received disadvantages. This interpretation seems unlikely, and instead we interpret the two-way root evolution as nonadaptive or nearly so and not as adaptive convergence (Rutishauser and Moline 2005). It is consistent with our findings that the foliose and ribbon-like species are sympatric, irrespective of phylogenetic relationships. Evolution of Podostemaceae appears to have occurred in the specific habitats that are monopolized by Podostemaceae and have little natural selection with competing macrophytes. This study may provide the first empirical data consistent with the idea of nonadaptive evolution of phenotypic characters in Podostemaceae (Willis 1914; see Introduction). However, data of microdistribution of sister species in the natural habitats yielded in this study are not detailed, and possibilities are not excluded that the derived ribbon-like rooted plants may occur in microhabitats different from those of the foliose plants in the same habitats and that each root form has both major advantages and minor disadvantages, with which the two root forms survive. Further studies are necessary, such as fitness analysis using experimental culture sets and an evo-devo study of the root forms.

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Author contributions SK and MK designed the study, conducted the research and wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Information on Electronic Supplementary Materials

Online Resource 1. Materials used in molecular phylogenetic analysis. Voucher data include localities, voucher and DDBJ accession numbers; locality data are omitted for vouchers with registered sequence data.

Online Resource 2. Alignment of *mat*K sequences used in phylogenetic analyses.

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