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New records, validated name, and redefinition in *Azolla* (Salviniaceae, Pteridophyta) from Costa Rica

Nuevos registros, nombre validado y redefinición en *Azolla* (Salviniaceae, Pteridophyta) para Costa Rica

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Abstract

In studies of herbarium specimens and living material, we observed that several species in the genus *Azolla* Lam. are present in different wetlands of Costa Rica, which instigated the search for applicable names of each taxon. According to a morphological and reproductive review, in addition to the species registered and validated, three species were recognized: *Azolla filiculoides* Lam., *A. imbricata* (Roxb. ex Griff.) Nakai and *A. pinnata* R. Br. The first species is the only native species from America, the second is from Asia, and the third is from Africa and Australia. The use of *Azolla* for fertilizing rice fields and animal feed has led to the introduction of species such as *A. imbricata* and *A. pinnata* to countries such as the United States, Colombia, and Ecuador. After their introduction, it is likely that aquatic and migratory birds were responsible for the spread of these species to other countries such as Costa Rica.

Keywords: invasive species, key, naturalized species, taxonomy, wetlands

Resumen

En los estudios de especímenes de herbarios y material vivo se observó que varias especies del género *Azolla* Lam. están presentes en diferentes humedales de Costa Rica, lo que implicó la búsqueda de los nombres aplicables a cada taxón. De acuerdo con la revisión morfológica y reproductiva, además de las especies descritas y validadas, se reconocieron tres especies: *Azolla filiculoides* Lam., *A. imbricata* (Roxb. ex Griff.) Nakai y *A. pinnata* R. Br. La primera especie es la única especie nativa de América, la segunda se conoce de Asia y la tercera se conoce de África y Australia. El uso de *Azolla* para la fertilización de arrozales y la alimentación animal ha hecho que especies como *A. imbricata* y *A. pinnata* se hayan introducido en países como Estados Unidos, Colombia y Ecuador; después de esto, probablemente las aves acuáticas y migratorias hayan sido las responsables de la propagación a otros países como Costa Rica.

Palabras clave: clave, especies invasoras, especies naturalizadas, humedales, taxonomía

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INTRODUCTION

The genus *Azolla* Lam. described with type *A. filiculoides* Lam., according to Moran (1995, 2004), is a floating aquatic fern with 1-2 mm leaves that is found in wetlands with little or no runoff, and is associated with the cyanobacteria *Anabaena azollae* Strasburger, 1884. According to Pereira et al. (2011), *Azolla* has bilobed leaves (dorsal and ventral lobes) that cover the entire rhizome. The *Azolla* species can be used as biofertilizer in rice culture, animal feed, and wastewater phytoremediation (Carrapiço et al., 2000; Lumpkin and Plucknett, 1980; Wagner, 1997).

Svenson (1944) made the first New World revision of *Azolla* species and used it as a principal character, the septate or unseptate glochidia, present in massulae (spore-masses) of microsporangia. The author recognized four species, species with unseptate glochidia are *A. caroliniana* Willd. and *A. filiculoides*. The former has small plants of 0.5-1 cm in diameter, but the latter has elongated plants 2-6 cm long. The species with septate glochidia are *A. mexicana* Schltdl & Cham. ex Kunze and *A. microphylla* Kaulf., the former with dichotomously branched plants and the later with pinnately branched plants.

Evrard and van Hove (2004) proposed a new classification for New World *Azolla* species with two species: *A. filiculoides* and *A. cristata* Kaulf. The authors mentioned that the former species has unicellular leaf trichomes, glochidia mainly unseptate or uniseptate, some with only a few apical septae, and its perine is warty. The latter species is characterized by bicellular leaf trichomes, glochidia mainly septate and a perine structure, quite variable, but not warty. Also, the name *A. cristata* for the second taxa based on the rule priority was used and not *A. caroliniana*.

Saunders and Fowler (1991) analyzed an *Azolla* section *Rhizosperma* and divided *A. pinnata* R. Br. into three subspecies, describing the subsp. *asiatica* R.M.K. Saunders and K. Fowler and combined subsp. *africana* (Desv.) R.M.K. Saunders and K. Fowler.

The phylogenetic analysis of *Azolla* used RAPD markers, the Jaccard similarity coefficient and UPGMA for the cluster analysis (Pereira et al., 2011), which allowed the separation of two large clades, the first contemplating the Old-World clade (*Rhizosperma* clade according to Svenson, 1944 and Tan et al., 1986) with *Azolla nilotica* Mett., *A. pinnata* var. *imbricata* (Roxb. ex Griff.) Bonap. and *A. pinnata* var. *pinnata*. The other clade known from American taxa and is divided into two clades: one containing *A. filiculoides* and *A. caroliniana*, and the other *A. microphylla* and *A. mexicana*.

Kay and Hoyle (2001) identified *Azolla pinnata*, and the taxa has been listed for sale in catalogs and is found in nurseries in several of the United States. Additionally, Madeira et al. (2013) mentioned that *A. pinnata* is present in Arthur R. Marshall Loxahatchee National Wildlife Refuge (in the northernmost portion of the Florida Everglades) and indicated that this species is spreading rapidly, causing concern that it may displace native *Azolla*. Aponte (2016) suggested the movement of aquatic plants was mainly by migratory waterfowl.

In addition to the use of *Azolla* in aquariums mentioned by Kay and Hoyle (2001), according to Ballesteros (2011), Méndez et al. (2018), Montaño (2005) and Wagner (1997), the application of *Azolla* as a biofertilizer in agricultural crops provides a natural source of the crucial nutrient nitrogen, and can also be used as an animal feed, a human food, a medicine, and a water purifier. *Azolla* is also used in the production of hydrogen fuel, the production of biogas, the control of weeds, the control of mosquitoes, and the reduction of ammonia volatilization which accompanies the application of chemical nitrogen fertilizer. This may explain why humans have taken *Azolla* to different parts of the world.

On the other hand, according to Madeira et al. (2013), *Azolla* has an invasive potential demonstrated by various worldwide species. Quick regeneration, rapid growth, broad distribution, and dense surface mats of *Azolla* can obstruct weirs, locks, and water intakes impeding irrigation, boating, fishing, and recreational activities (Baars and Caffrey, 2010; Hashemloian and Azimi, 2009; McConnachie et al., 2003; Rivera, 2020). A dense surface cover may also reduce aquatic oxygen levels (Janes et al., 1996) and submerge animal populations (Gratwicke and Marshall, 2001). For this reason, it represents a problem in natural ecosystems.

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MATERIALS AND METHODS

Different taxa of *Azolla* observed in natural habitats were recollected and preserved according to the guide of Lorea and Riba (1990). The identification of species was made principally with the use of the papers published by Evrard and Hove (2004), Saunders and Fouler (1992) and Svenson (1944). The specimens collected were deposited in the herbaria: CR, MO and K, acronyms following Holmgren (1998). For morphology and reproductive structures, species were cultivated in the Natural Resources and Wildlife Laboratory (LARNAVISI). Plants were cultivated in 1.5 x 1.5 m ponds less than 0.5 m deep, enriched with chicken manure and had access to high solar radiation. Plants were cultivated in 1.5 x 1.5 m ponds less than 0.5 m deep, enriched with chicken manure and had access to high solar radiation, given ideal conditions, the plants were allowed to grow until they reached a high level of competition among themselves, inducing reproduction by spores. Subsequently, plants were studied and photographed using a microscope (Olympus LX31 and Olympus BX41 HD) and stereo microscope (Motic SMZ-168 series and Optika ST-30FX). Cameras used were those from a Samsung Galaxy note 10 and Samsung Galaxy A50 telephones.

To ensure the correct application names, original type material or digital type images were examined as available (Jstor Global Plants (http://plants.jstor.org/), and the correct use of scientific names and authors is according to the International Plant Name Index (http://www.ipni.org/ipni/plantnamesearchpage.do). The distribution of specimens was corroborated in the Tropicos Database (Missouri botanical Garden, 2016).

RESULTS

After the morphologic and reproductive studies of *Azolla* specimens from Costa Rica, three species were recognized: *Azolla filiculoides*, *A. imbricata* and *A. pinnata*. *Azolla filiculoides* is the only species native to America and is characterized by dense glochidia in the massules of spores. The latter two species are new records from Costa Rica, as species scaped of cultivation and naturalized in natural ecosystems.

Redefinition of species

Azolla filiculoides Lam., Encycl. 1(1): 343. 1783.

Type. Argentina: Straits of Magellan, *Commerson* s.n. (P!, MPU!).

Synonyms. *Azolla cristata* Kaulf., Enum. Filic. 274. 1824. Type. America meridioa, *Otterband* s.n. (P!).

Azolla caroliniana Willd., Sp. Pl. 5(1): 541. 1810. Type. USA (Carolinas), Habitat in aquis Corolinae, *Richard* s.n. (B!).

Azolla mexicana Schltdl. & Cham. ex Kunze, Linnaea 18(3): 352. 1844[1845]. Type. *Leibold* 150 (?).

Azolla microphylla Kaulf., Enum. Filic. 273. 1824. Type. USA: "California", *Chamisso* s.n. (P!).

Diagnostic characters. Plants 0.5-1.5 (-2) cm long (rarely longer) in their mature state, dichotomously branched; adaxial lobes 0.7-1.2 mm long, ovate, covered with trichomes 0.1-0.2 mm long; abaxial hyaline lobes with cells quadrangular to rectangular, the cells 1.5-3 times longer than wide; massules of microspores cover by dense glochidia with blunt apex and side hooks (figures 1, 2 and 3).

Taxonomic discussion. Pereira et al. (2011) obtained two clades using 13 polymorphic vegetative characters, one including *Azolla filiculoides*, *A. microphylla* and *A. rubra*, but it is known that the definition of *A. rubra* R. Br. is incorrect because all (except *A. nilotica* Mett.) can develop a red color in their leaves under stressful conditions. The other clade is formed by *A. caroliniana* and *A. mexicana*. However, in the cladogram derived from the analysis of 211 polymorphic RAPD markers, the result is contradictory to the previous information, because *A. filiculoides* forms a clade joined with *A. caroliniana* and the other clade is formed by *A. mexicana* and *A. microphylla*. Further, a specimen of *A. caroliniana* appeared mixed with *A. filiculoides* species, and a specimen of *A. microphylla* appeared mixed with *A. caroliniana* species. Additionally, Evrard and Van Hove (2004) mentioned that in 29 papers that they found, *A. filiculoides* is characterized by glochidia mainly unseptate or uniseptate, sometimes accompanied by glochidia with two or three septae, and other species are characterized mainly pluriseptate glochidia, but clearly this is not an exclusionary

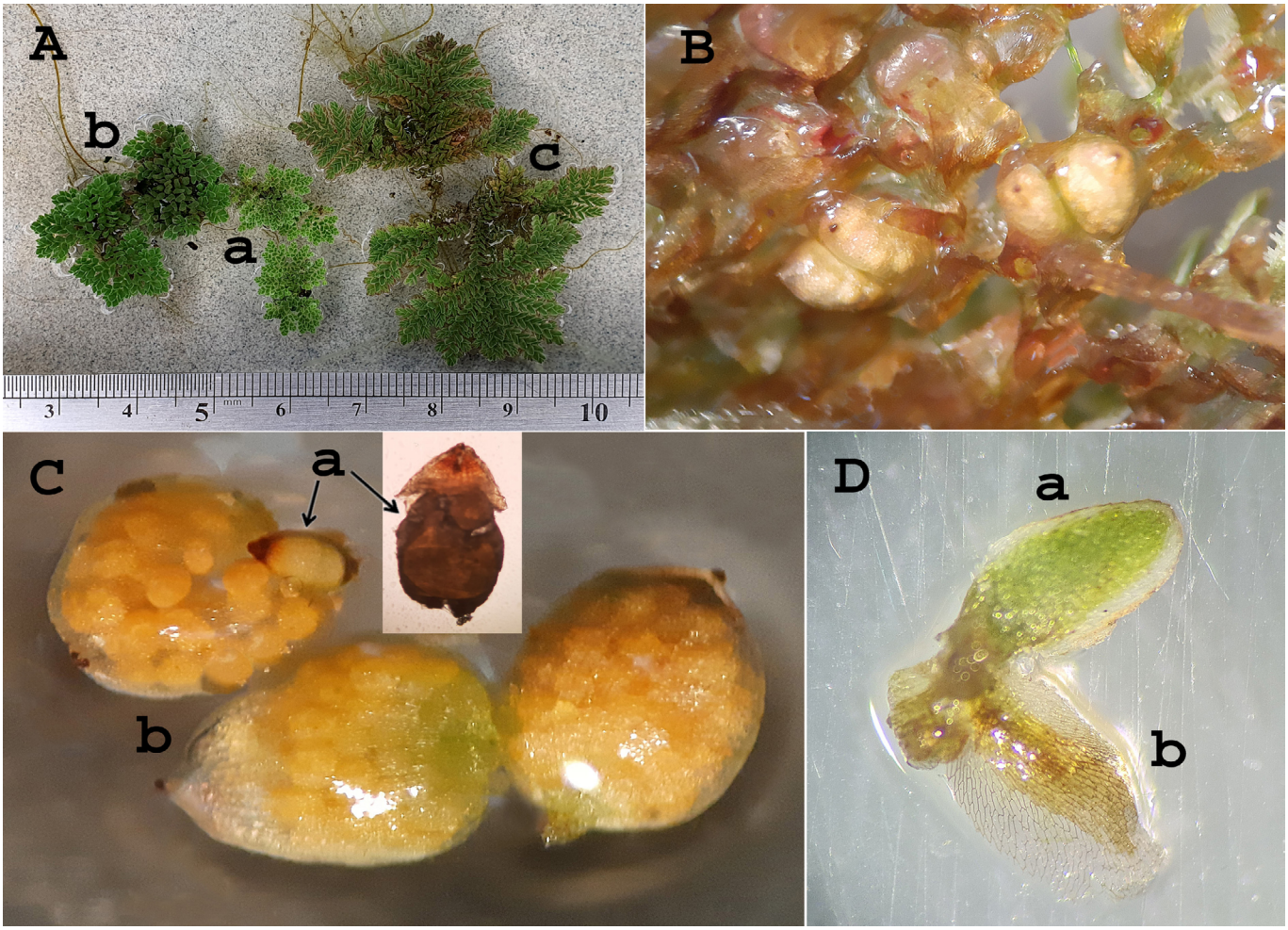

Figure 1. A. Comparative habit of the three *Azolla* species present in Costa Rica: **a.** *Azolla filiculoides* Lam. **b.** *A. imbricata* (Roxb. ex Griff.) Nakai and **c.** *A. pinnata* R. Br. **B.** Abaxial surface of a *A. pinnata* plant showing paired microsporocarps. **C.** *A. pinnata* comparative size of **a.** megasporocarps and **b.** microsporocarps. **D.** *A. pinnata* showing **a.** chlorophyllic adaxial lobe and **b.** hyaline abaxial lobe.

characteristic. Also, according to the previous information it is better to use only one species name for the American *Azolla.*

New records and validated name

Azolla imbricata (Roxb. ex Griff.) Nakai, Bot. Mag. (Tokyo) 39(463): 185. 1925.

Basionym. *Salvinia imbricata* Roxb. ex Griff., Calcutta J. Nat. Hist. 4: 469. 1844.

Lectotype. India. Bengal: W. Rosburgh s.n. (BR!; isolectotype: BM!).

Synonym. *Azolla pinnata* subsp. *asiatica* R.M.K. Saunders and Fowler, Bot. J. Linn. Soc. 109(3): 349-351. 1992. Type: Thailand. North Phayao, 2 Mar 1958, T. Sorensen, K. Larsen and B. Hansen 1829 (K).

Diagnostic characters. Plants up to 3 cm long; adaxial lobes patent to slightly imbricate; abaxial hyaline lobes shorter and wider (the relation of approx. 1.2:1, length:width), with cells principally rectangular; massules 4 (-5) per sporangia, they with three to six basal glochidia (figures 1, 2 and 3).

Known distribution. Bangladesh, Burma, China, India, Indonesia, Japan, Malaysia, North Korea, Pakistan, Philippines, South Korea, Sri Lanka, Thailand and Vietnam.

Material of new distribution. COSTA RICA. Limón: Talamanca, Sixaola, Gandoca, Lagunas 2 y 3 al S. de Gandoca, 9°35'00" N, 82°35'00" W, 0-5 m. s. n. m., 18 sep 2022, A. Rojas and M. Obando 12524 (CR, K, MO, USJ) (figures 4 and 5).

Taxonomic discussion. According to Pereira et

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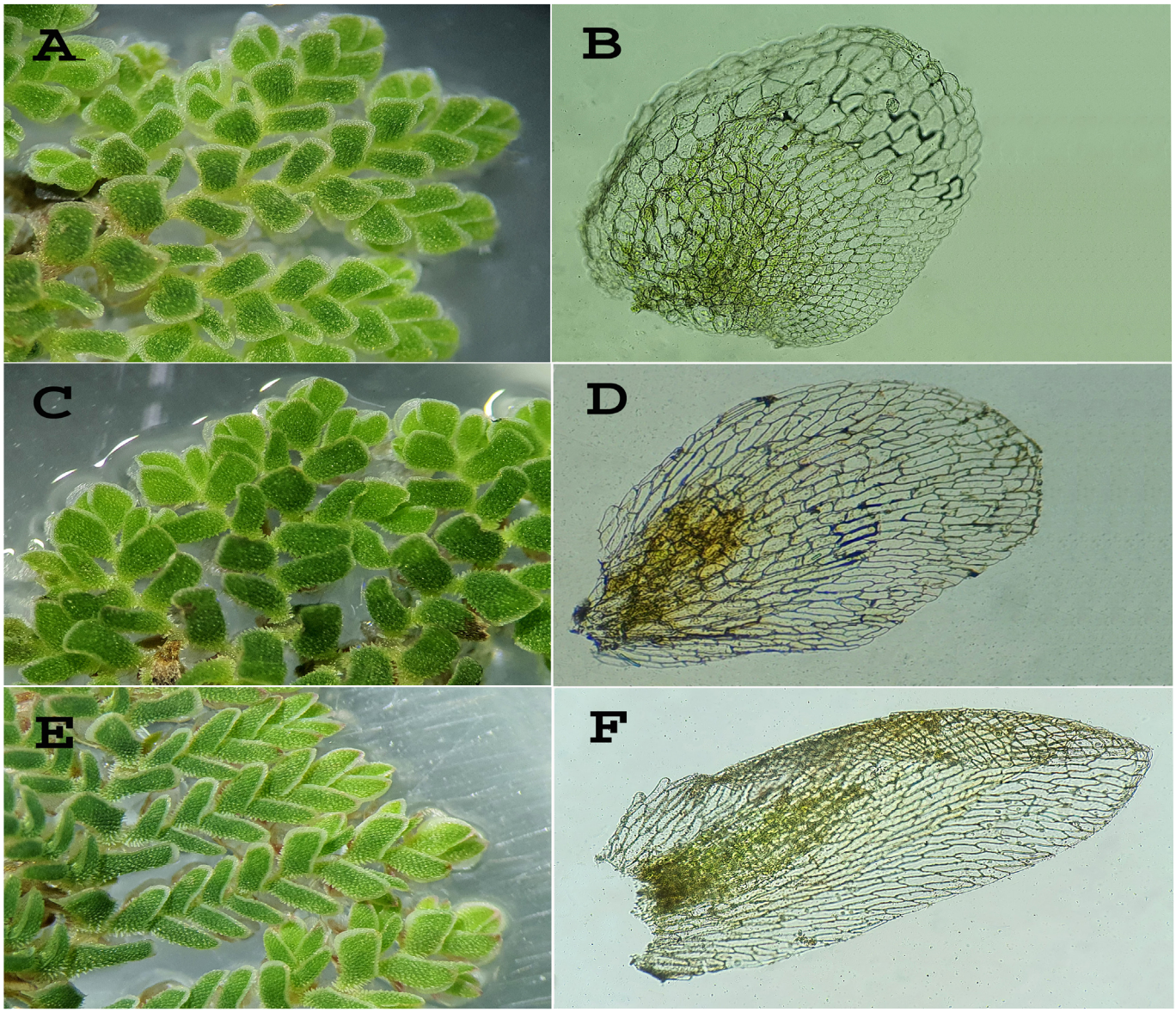

Figure 2. A and B. *Azolla filiculoides* Lam. **A.** Adaxial detail of plant showing chlorophyllic lobes. **B.** Abaxial hyaline lobe showing cells. **C and D.** *A. imbricata* (Roxb. ex Griff.) Nakai: **C.** Adaxial detail of plant showing chlorophyllic lobes. **D.** Abaxial hyaline lobe showing cells. **E and F.** *A. pinnata* R. Br.: **E.** Adaxial detail of plant showing chlorophyllic lobes. **F.** Abaxial hyaline lobe showing cells.

al. (2011) the cladogram derived from the analysis of 211 polymorphic RAPD markers shows a first clade containing all the Old World *Azolla*, which is divided into two clades, one for *A. nilotica*, and the other one with *A. pinnata*. The second *A. pinnata* clade is divided into two clades: var. *imbricata* (Roxb. ex Griff.) Bonap. and var. *pinnata*. The above information contradicts the taxonomic classification made by Saunders and Fowler (1992), where they divided *A. pinnata* into three subspecies: subsp. *asiatica* R.M.K. Saunders and K. Fowler, subsp. *africana* (Desv.) R.M.K. Saunders and K. Fowler and subsp. *pinnata*. The previous information is combined with the morphological and reproductive characteristics that we found. The two clades mentioned by Pereira et al. (2011) are interpreted here as *A. imbricata* and *A. pinnata*.

Azolla pinnata R. Br., Prodr. 167. 1810.

Type. Australia. Hawkesbury, Richmond, R. *Brown* 134 (Lectotype: BM!; Isolectotypes: E, K!, MEL!). Lectotype designed by R.M.K. Saunders and K. Fowler (1992).

Synonyms. *Azolla africana* Desv., Mém. Soc. Linn. Paris 6(2): 178. 1827. Type: Africa. *Anon.* s.n. (herb. spec. P00483231) (P!).

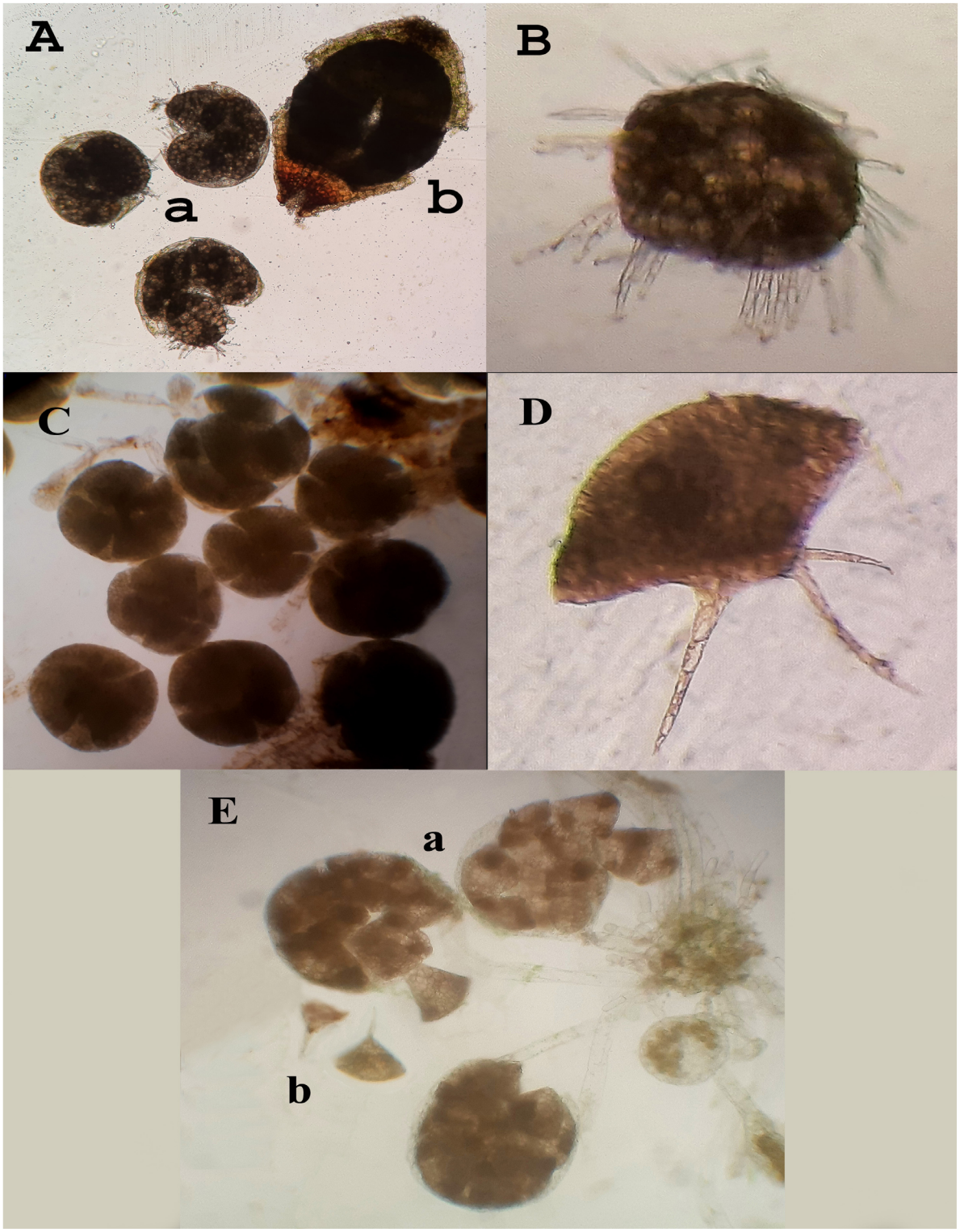

Figure 3. A and B. *Azolla filiculoides* Lam.: **A.** Microsporangia **(a)** and Megasporocarp **(b). B.** Massule with glochidia all around. **C and D.** *A. imbricata* (Roxb. ex Griff.) Nakai: **C.** Microsporangia. **D.** Massule with basal glochidia. **E.** *A. pinnata* R. Br.: Microsporangia **(a)** and Massules with one basal glochidia **(b).**

Diagnostic characters. Plants to 6.5 cm long; adaxial lobes imbricate; abaxial hyaline lobes longer and narrower (the relation of approx. 1.7:1, length:width), with cells principally trapezoidal to rhomboidal; massules (7-) 8-10 per sporangia, with one basal glochidia (figures 1, 2 and 3).

Known distribution. Australia, Gambia, Senegal, Kenya, Tanzania, Chad, Sudan, Botswana, Madagascar, South Africa and United States of America (naturalized).

Material of new distribution. COSTA RICA.

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Heredia: Sarapiquí, Orquetas, asentamiento La Chaves, parcela 48, propiedad de Adita Castro, 10.383445° N, 85.948773° W, 60 m. s. n. m., 23 Jul 2010, J. González 11172 (LSCR). Puntarenas: cantón de Aguirre, carretera a Quepos, entre Palo Seco y Damas, 9°31'43" N, 84°15'52" W, 5 m. s. n. m., 14 Jul 2022, A. Rojas and M. Obando 12478 (CR, K, MO, USJ) (figures 4 and 5).

Taxonomic discussion. See discussion under *Azolla imbricata.*

Key for the world species of *Azolla* (figures 1, 2 and 3).

1. Plants elongate, 20-50 cm long, never red; ventral lobe of fronds more than one cell thick and chlorophyllous with stomata and trichomes; sporocarps grouped in fours.....*A. nilotica*

1'. Plants deltoid to trapezoidal, 0.5-6.5 cm long, red under adverse environmental conditions; ventral lobe of fronds of one cell thick and translucent, lacking stomata and trichomes; sporocarps grouped in twos mainly.....2

2. Plants 0.5-1.5 (-2) cm long (rarely longer) in its mature state, dichotomously branched; adaxial lobes 0.7-1.2 mm long, ovate, cover with trichomes 0.1-0.2 mm long; abaxial hyaline lobes with cells quadrangular to rectangular, the cells 1.5-3 times longer than wide; massules of microspores cover by dense glochidia with blunt apex and side hooks.....*A. filiculoides*

2'. Plants 2-6.5 cm long in its mature state, pinnately or irregularly branched; adaxial lobes 1.3-1.7 mm long, oblong to lanceolate, cover with trichomes 0.2-0.5 mm long; abaxial hyaline lobes with cells rectangular to rhomboidal, the cells 4-10 times longer than wide; massules of microspores 1-6 basal glochidia with acute tip but without side hooks.....3

3. Plants to 3 cm long; adaxial lobes patent to slightly imbricate; abaxial hyaline lobes shorter and wider (the relation of approx. 1.2:1, length:width), with cells principally rectangular; massules 4

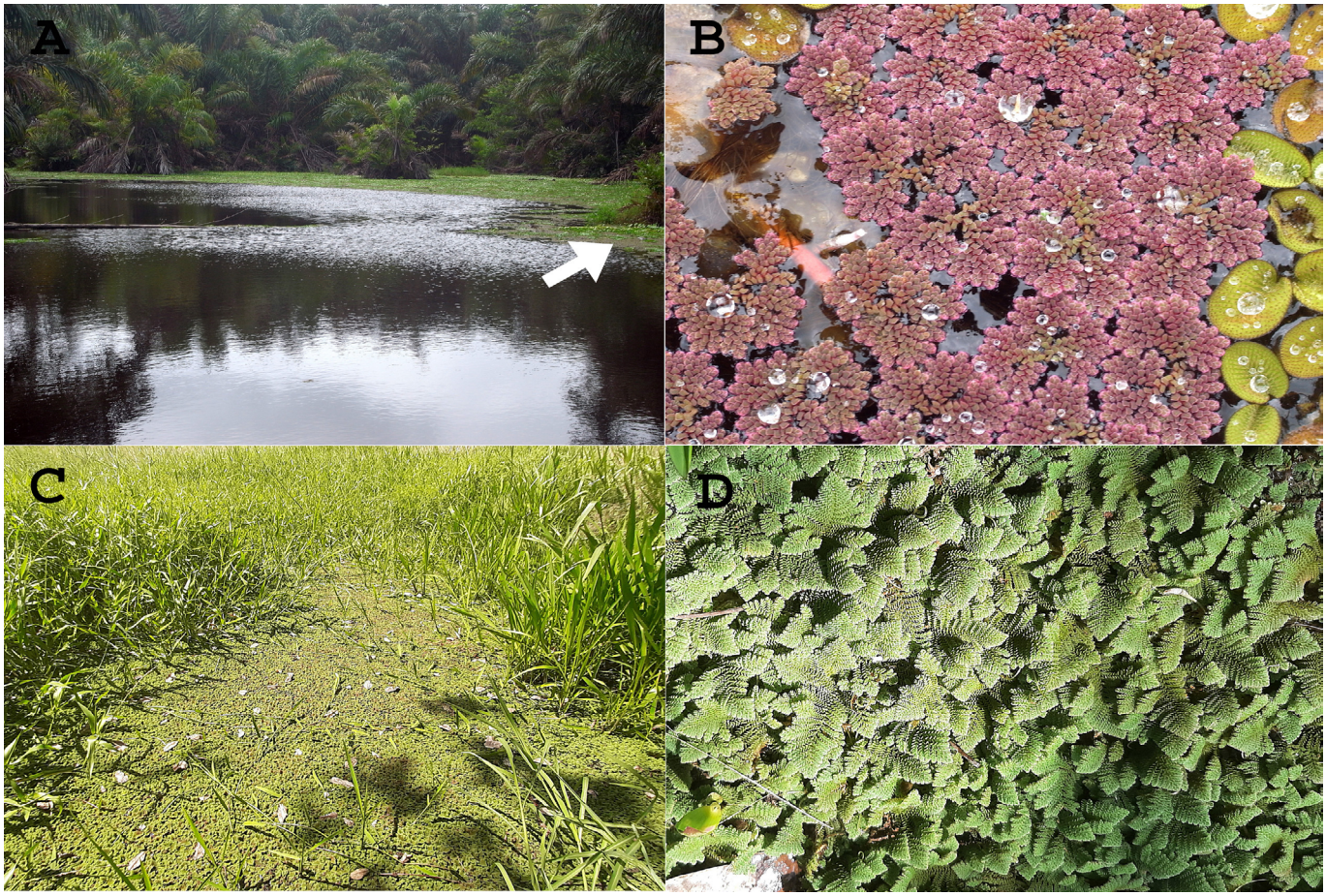

Figure 4. A and B. *Azolla imbricata* (Roxb. ex Griff.) Nakai invading natural habitat in Limón, Talamanca, Sixaola, Gandoca, wetlands 2 and 3. **C and D.** *Azolla pinnata* R. Br. invading natural habitat in Puntarenas, Aguirre, between Palo Seco and Damas, Costa Rica.

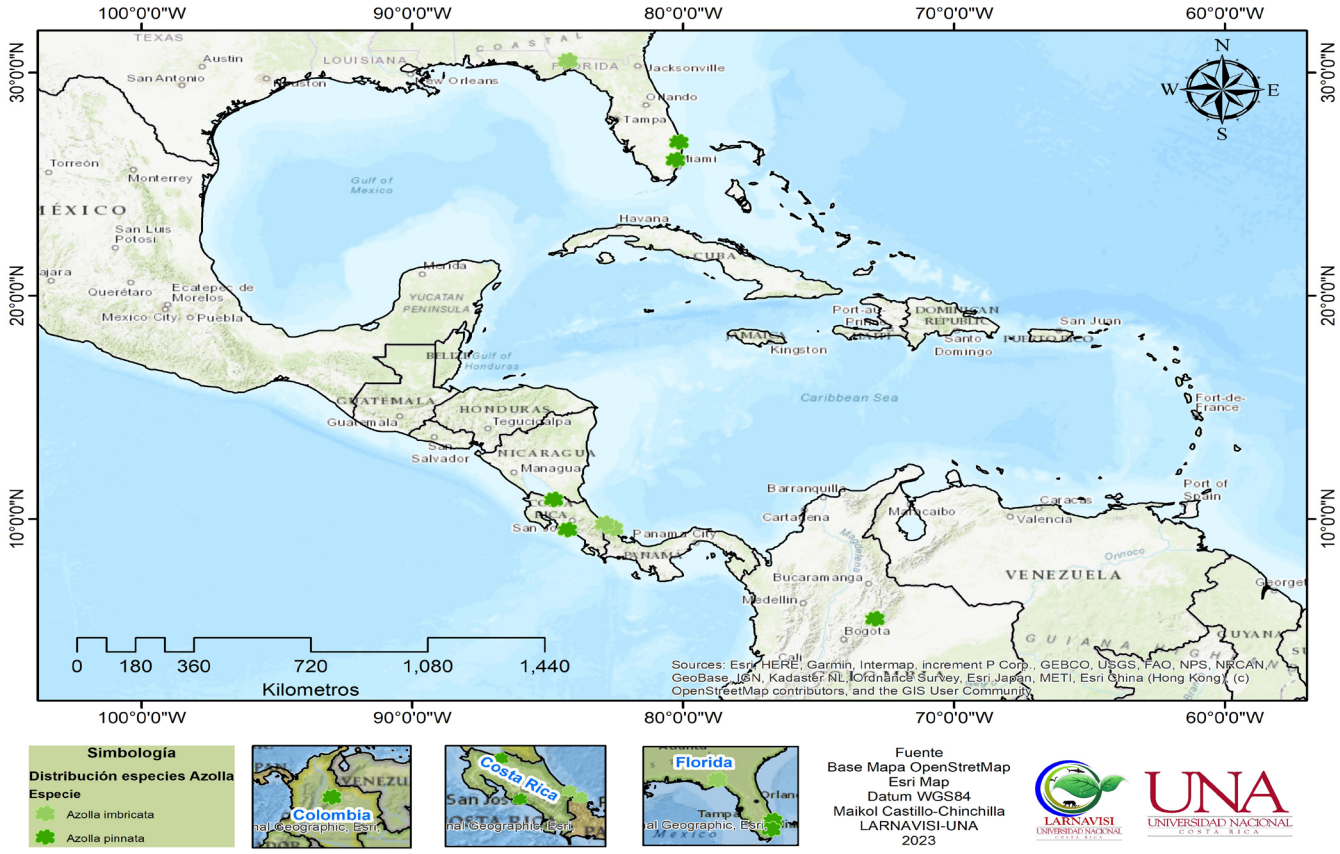

Figure 5. Distribution map of *Azolla imbricata* (Roxb. ex Griff.) Nakai (indicated with a pale green asterisk) and *A. pinnata* R. Br. (indicated with a dark green asterisk), based on our records and those mentioned in Gbif: https://www.gbif.org/es/species/ 2650106 which have been corroborated by other sources.

(-5) per sporangia, they with three to six basal glochidia.....*A. imbricata*

3'. Plants to 6.5 cm long; adaxial lobes imbricate; abaxial hyaline lobes longer and narrower (the relation of approx. 1.7:1, length:width), with cells principally trapezoidal to rhomboidal; massules (7-) 8-10 per sporangia, they with one basal glochidium.....*A. pinnata*

DISCUSSION

According to Evrard and Van Hove (2004) the American species of *Azolla* can be distinguished by the septate or unseptate glochidia present in massulae of microspores, the number of cells in the trichomes present in the abaxial surface of the blade and perine of spores; all characteristics that can be observed only with electron microscopy. In this paper, two species are recorded: *A. imbricata* and *A. pinnata*, and only one species is validated from America: *A. filiculoides*, a key is offered for all species that uses macroscopic and microscopic characters that can be seen with a stereoscope, leaving in the background characters of reproductive structures that are not always present in plants.

The presence of alien species in natural systems is one of the main problems caused by global change (Sala, 2000). The *Azolla* species studied can make their incursion by various routes such as the mass transport of people and goods, aquariums or their cultivation to fertilize rice plantations; the latter two being the most likely causes of their introduction.

In countries such as Cuba, Ecuador and Bangladesh, the use of the genus *Azolla* as a biofertilizer has been promoted as a mechanism to alleviate the energy crisis and the high cost of chemical fertilizers (Castro et al., 2002; Montaño, 2005), as a feed supplement in aquaculture and livestock (Biplob et al., 2002; Méndez-Martínez et al., 2018), biomass production (Rivera, 2020) and in water treatment to reduce salts and heavy metals (Ballestero, 2011; Batalla and Serrano, 2016). This could have led to the introduction of species in the Neotropics, their use in cultivated areas and their incursion

into natural environments.

Being that introduction to wetlands can be mainly due to the activity of birds and other animals that can act as vectors to disperse these species. The establishment of these ferns can affect native plant communities and people (Lockwood et al., 2009; Sala, 2000; Simberloff et al., 2013) because they can compete and cause local extinction and alter ecosystems.

In Spain, it is possible that the introduction of *Azolla filiculoides* into natural environments was caused by the movement of birds that migrate between wetlands from Portugal, where it has been established since 1920 (Sanz Elorza et al., 2004). For example, in the Doñana National Park it has been suggested that this species came from nearby wetlands, transported by birds, by contact between water masses, or translocations of birds that came from localities where this fern was introduced (Cobo, 2002). It has also been reported in Segovia (Spain) that the migration of waterfowl combined with the sexual and asexual reproduction of *Azolla* has facilitated their expansion in natural environments (Martín, 2010). This same behavior may be what favored the naturalization of this genus in Costa Rica.

For their part, mammals have played a clear role in the dispersal of seeds, spores, and even complete individuals of ecosystems, because they consumed it and later excreted it, or it adhered to their hair and was thus transported, in addition to the ability to move between different ecosystems including wetlands (Mora Cabeza et al., 2015). Therefore, it is possible that species of this group also facilitate dispersal in natural environments. Added to the observation, carried out in our cultivation ponds of transport carried out by raccoons (*Procyon lotor*), domestic rats (*Rattus norvegicus f. domestica* and *Rattus rattus*) and house mice (*Mus musculus*).

Given its importance in various economic activities and its capacity for dispersion through various means, it is essential to propose management measures to reduce the risk of invasion, especially in protected wild areas according to the IUCN (2019).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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