ARTICLE HTTP://DX.DOI.ORG/10.5504/BBEQ.2013.0088 A&EB

PERSICARIA AMPHIBIA, A SERIOUS TERRESTRIAL WEED IN NORTHERN GREECE: A COMBINED MOLECULAR AND MORPHOLOGICAL APPROACH TO IDENTIFICATION AND TAXONOMY

Thomas K. Gitsopoulos[1], Ioannis Vasilakoglou[2] and Georgios Tsoktouridis[3]
[1]Hellenic Agricultural Organization "Demeter", Plant Protection Institute of Thessaloniki, NAGREF, Thessaloniki, Greece
[2]Technological Educational Institute of Thessaly, Department of Agricultural Technology, Larissa, Greece
[3]Hellenic Agricultural Organization "Demeter", Laboratory for the Conservation and Evaluation of Native and Floricultural Species, Thessaloniki, Greece.
Correspondence to: Georgios Tsoktouridis
E-mail: gtsok1@yahoo.co.uk

ABSTRACT

The versatile rhizomatous terrestrial form of the perennial weed Persicaria amphibia is reported to cause severe infestations in potato crops, which are mainly cultivated as a monoculture in the area of Kato Nevrokopi located at the Greek–Bulgarian boundaries. This weed cannot be easily eradicated from potato crops in Northern Greece and therefore, the accurate taxonomic identification of P. amphibia has a high economical importance. The generic classification of P. amphibia has been unstable, mostly due to different morphological interpretations with other Persicaria species. Molecular studies based on DNA sequence data from the rbcL and matK plastid regions supported 100 % homology with P. amphibia. Further analysis using the psbK-psbI, atpF-atpH, rpoC1 and rpoB plastid regions identified distinct differences with other Persicaria species suggesting the application of psbK-psbI and atpF-atpH as potential molecular markers for identification and phylogenetic analysis of Persicaria taxa. Morphology description and ecology characteristics concerning the terrestrial form of P. amphibia are provided.
A multidisciplinary approach with both molecular and morphological information is described in this study and provides potential useful tools for the identification and taxonomy of Persicaria species.

Biotechnol. & Biotechnol. Eq. 2013, **27**(6), 4236-4242

Keywords: potato crop, molecular identification, morphology, classification, chloroplast DNA, Polygonaceae

Introduction

The weed species *Persicaria amphibia* (L.) Delarbre or *Polygonum amphibium* (L.) Gray (20, 41) with common name 'water knotweed', 'water smartweed', or 'amphibious bistort' is a versatile rhizomatous perennial that is described for both aquatic and terrestrial growth forms (37). These forms are so different that they could be mistaken for different species (30). The plant belongs to Polygonaceae Juss. family, which as reported by Rai et al. (36), is a cosmopolitan family including 48 genera and about 1200 species (28, 38). All these plants are characterized by the presence of the ochrea, a formation on the main stem that derives from the fusion of the stipules (36). The taxonomy of this family is complex (6) with high phenotypic variability (2).

Although morphological and ecological characters are used for identification and taxonomy, many of the species are difficult to be classified, due to their high variability, resulting in greater taxonomical confusion (22). Moreover, other authors also stated that *Persicaria* plants are themselves highly variable in morphology (9, 13, 31). Concerning *P. amphibia*, Mitchell (32) reviewed its mutability growth-forms in terms of environmental and genetic influences. Partridge (35) reported that the alternative nomenclature of the plant, *Polygonum amphibium* L. has been included in the Section *Persicaria* (Miller) DC. of *Polygonum* L. s.l. (Fl. Europ. 1, 2nd edn). The genus *Persicaria* Miller was considered sufficiently distinct to be of generic rank (6, 8, 16). Partridge (35) also reported the recognition of *P. amphibium* L. var. *amurense* Korsh. (Fl. USSR V) with white-flowered plants and cordate leaves from North-West China and far-Eastern Russia, while in North America there are two currently recognized endemic varieties of *P. amphibium* L. var. *stipulaceum* Coleman and var. *emersum* Michx. Although the former has flared stipular appendages (32), the distinctions are subtle.

Plant DNA barcodes have been proved extremely useful for numerous applications such as ecological forensics, identification of traded materials, undertaking identifications where there is a shortage of taxonomic expertise available and assisting species discovery in some plant groups (18). The role of genetic data from chloroplast DNA (cpDNA) can be clearly seen in the fields of plant phylogenetics, systematics, population genetics and molecular biology (34). Four main suggestions for a plant barcode have been proposed for the suitability of various coding and non-coding cpDNA markers (11, 25). These are *rpoC1+rpoB+matK* or *rpoC1+matK+trnH-psbA* (5), *rbcL+trnH-psbA* (24) and *atpF-H+psbKI psbKI+matK* (21). However, Lahaye et al. (27) proposed that *matK* alone should constitute the plant barcode. Among the two-locus barcode combinations, *rbcL_matK* was the majority

choice for several reasons. High-quality sequences of *rbcL* are easily retrievable across phylogenetically divergent lineages, and it performs well in discrimination tests in combination with other loci. This combination represents a pragmatic solution to a complex trade-off between universality, sequence quality, discrimination, and cost (4). Developing amplification strategies for *matK* was considered an investment with better prospects for return than solving the problem of sequence quality in *trnH–psbA* caused by mononucleotide repeats (7).

The key foundation step for plant barcoding is in reaching agreement on a standard set of loci to enable large-scale sequencing and the development of a global plant barcoding infrastructure (4). Sequencing *rbcL* and *matK* as a standard 2-locus barcode, is thus an important step in establishing a centralized plant barcode database as a tool for taxonomy, conservation, and the multitude of other applications (33) that require identification of plant material.

Molecular phylogenetic studies on Polygonaceae have supported the molecular classification of *Persicaria* species and subspecies based on *psbA-trnH*, *psaI-accD*, *matK*, *ndhF* and *rbcL*, nuclear ribosomal DNA (ITS), (3), *trnL-F* and ITS (44), *rbcL* (12), *rbcL*, *trnL–F*, *trnK* intron–*matK*, *psbA–trnH* IGS and ITS (22) and the *LEAFY* second intron (23).

Persicaria amphibia has always been related to all the other species of sect. *Persicaria*, nevertheless it has some characteristics (pollen typology and the presence of a robust rhizome) which place it in a particular position (12). The analyses of some nucleotide sequences by Kim and Donoghue (22) and Kwak et al. (26) confirm its isolated position.

The weed recorded in Kato Nevrokopi, Greece, is observed in its terrestrial form and has become a serious weed difficult to control in potato fields, the main crop of the locality. The terrestrial form of this weed may be mistaken with other taxa of similar morphology. More specifically, Partridge (35) reported that the vegetative terrestrial form of *P. amphibia* may be mistaken with *P. maculosa* L. In addition, *P. amphibia* has been recently characterized as a problematic species in the Netherlands (43).

In this study, we provide a methodology for identification and taxonomy of *Persicaria amphibia*, based on morphology and molecular classification using DNA sequences of the *matK*, *psbK-psbI*, *atpF-atpH, rpoC1*, *rpoB* and *rbcL* plastid regions. These molecular markers are six of the seven ones recommended by the CBOL Plant Working Group (4), as a universal framework for the routine use of DNA sequence data to identify specimens and contribute toward the discovery of overlooked species of land plants. The seventh one is the *trnH–psbA* plastid region that does not consistently provide bidirectional unambiguous sequences, often requiring manual editing of sequence traces and, therefore, it has been excluded in this study. In summary, *rbcL* offers high universality and good, but not outstanding discriminating power, whereas *matK* offers higher resolution, but requires further development work. Thus, no single locus meets CBOL's data standards and guidelines for locus selection, and as a result, a synergistic combination of loci is required. In the present study, the *rbcL* and *matK* gene regions provide sequence information in GenBank for the identification of *Persicaria amphibia* voucher 2012PolAm_Nevrokopi.

Materials and Methods

Morphology

The morphological description, molecular studies and plant analysis of *P. amphibia* were based on plants collected from several potato fields of Kato Nevrokopi geographic region (Elev: 509 m, Lat: 41° 18′ 38′′ N, Long: 23° 49′ 36′′ E) one of the most important potato cultivation areas of Greece. This area is an isolated plateau surrounded by mountains. The herbs were collected in the beginning of August 2012 at the flowering vegetative growth stage. The plant tissue element analysis was performed according to Jones (19); three leaves of medium size, two stems and two rhizomes 10 cm in length were collected from each plant.

Plant material and DNA extraction

Genomic DNA was extracted from fresh young leaves, which were first ground and homogenized using liquid nitrogen in a mortar with a pestle. Then, DNA was extracted with a Plant DNA Extraction kit from Qiagen (Germany), according to the protocol provided by the manufacturer. The DNA produced was quantified in an Eth-Br Agarose (0.6 %) gel in TAE electrophoresis buffer (pH 8.5) at room temperature, using known amounts of λ DNA in comparison and visualized on a UV transilluminator.

Oligonucleotide primers

The oligonucleotide primers synthesized for this research were purchased from InVitrogen Inc. (U.K.). A number of oligonucleotide stock primers that are routinely used for plant DNA barcoding in various projects in the molecular Laboratory for the Conservation and Evaluation of Native and Floricultural Species were successfully selected to amplify the *matK*, *psbK-psbI*, *atpF-atpH, rpoC1*, *rpoB* and *rbcL* plastid regions of *P. amphibia* samples. Specifically, the forward 5'- CTYGAACCCGGARCTNRTCGGA -3' primer designed by the authors and the reverse one 5'-ACCCAGTCCATCTGGAAATCTTGGTTC-3' by CBOL Plant Working Group (4) were utilized to amplify an ~1200 bp PCR product of the *matK* region. The forward 5'- TTAGCCTTTGTTTGGCAAG -3' and the reverse 5'-AGAGTTTGAGAGTAAGCAT-3' oligonucleotide primers (4) were utilized to amplify an ~450 bp PCR product of the *psbK-psbI* region. The forward 5'-ACTCGCACACACTCCCTTTCC-3' and the reverse 5'-GCTTTTATGGAAGCT TTAACAAT-3' oligonucleotide primers (4) were utilized to amplify an ~600 bp PCR product of the *atpF-atpH* region. The forward 5'-GGCAAAGAGGGAAGATTTCG-3' and the reverse 5'-CCATAAGCATATCTTGAGTTGG-3' oligonucleotide primers (10) were utilized to amplify

an ~550 bp PCR product of the *rpoC1* region. The forward 5'-AAGTGCATTGTTGGAACTGG-3' and the reverse 5'-GATCCCAGCATCACAATTCC-3' oligonucleotide primers (10) were utilized to amplify an ~500 bp PCR product of the *rpoB* region. The forward 5'-ATGTCACCACAAACAGAGACTAAAGC-3' and the reverse 5'-CTTCTGCTACAAATAAGAATCGATCTC-3' oligonucleotide primers (25) were utilized to amplify an ~600 bp PCR product of the *rbcL* region.

PCR amplification

PCR amplification was carried out using a DNA thermal cycler (Eppendorf, Germany), programmed to denature DNA at 95 °C for 15 s, anneal at 60 °C and extend at 72 °C for 1 min, for 35 cycles in a 50 µL mixture containing 2 U of *Taq* DNA polymerase (GeneOn, Germany), 200 µmol/L of each deoxynucleoside triphosphate (HT Biotechnology Ltd., UK), 1.8 mmol/L $MgCl_2$, 1 pmol of each oligonucleotide primer, 10 ng to 50 ng of DNA template and 10x Mg^{++}-free Buffer (670 mmol/L Tris-HCl, pH 8.8, 166 mmol/L $(NH_4)_2SO_4$ and 0.1 % Tween 20). An initial 4 min denaturation at 95 °C and a final 10 min extension at 72 °C were included in the program. After amplification, 6 µL of DNA loading buffer (20 % glycerol, 5 mmol/L EDTA, bromophenol blue) was added and 16 µL of the PCR reaction mixture separated by electrophoresis through an Eth-Br Agarose (1.5 %) gel, in TAE electrophoresis buffer (pH 8.5) at room temperature and viewed on a UV transilluminator. A single PCR product was obtained for each of the above molecular markers. Purification was done using a PCR Spin Column Purification System (Macherey-Nagel, Germany) and then quantification in an Eth-Br Agarose (1.2 %) gel in TAE electrophoresis buffer (pH 8.5) at room temperature, using known amounts of a 100 bp DNA ladder (GeneOn, Germany) in comparison. The products were visualized on a UV transilluminator.

Sequence analysis

The amplified PCR products of *matK*, *psbK-psbI*, *atpF-atpH, rpoC1*, *rpoB* and *rbcL* plastid regions were directly sequenced by Macrogen (Korea). Every PCR product was sequenced once at both directions using the amplification primers. Sequence data were analyzed using the EditSeq, SeqMan and MegAlign programs from the DNA-Star Nucleotide Sequence Analysis Package. Sequences were blasted at the GenBank database

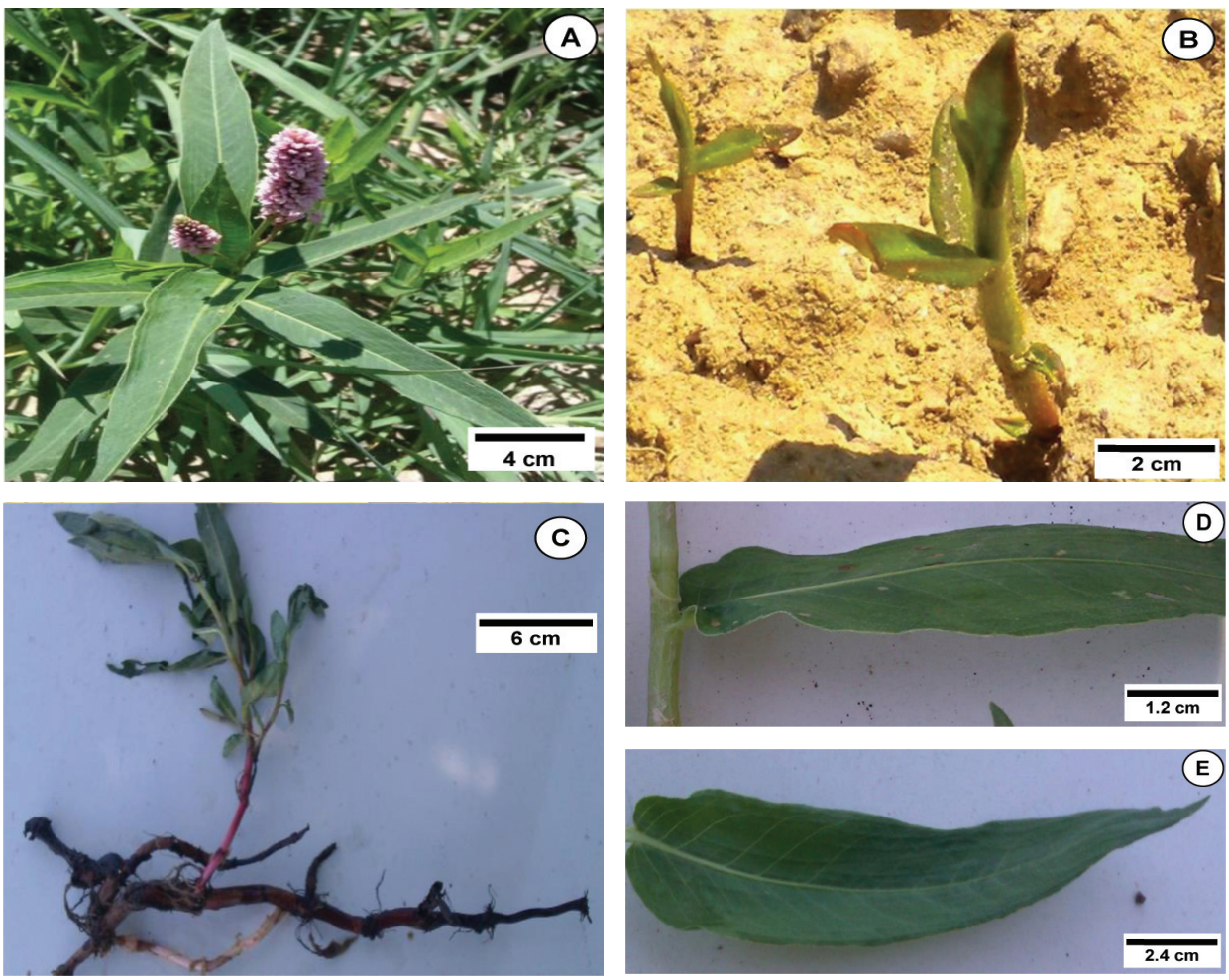

Fig. 1. *Persicaria amphibia* voucher 2012PolAm_Nevrokopi: plant with two inflorescences(**A**); young plants emerged from rhizomes (**B**); rhizome and young plant (**C**); ochrea length and leaf (**D**); leaf length, rounded base and acute apex (**E**).

using the "nblast" algorithm (1) and manually edited using EditSeq to reduce mis-aligned regions. Sequences were aligned according to the ClustalW method using the equations described by Thompson et al. (42) and phylogenetic dendrograms were obtained for the *matK* and *rbcL* genes. Due to poor alignment and incomplete sequences at the beginning and the end of the alignment, characters were excluded from analysis and we focused on the conserved plastid regions generated in this study (Sequences of *P. amphibia* 2012PolAm_Nevrokopi with accession numbers KC342450-5).

Results and Discussion

Morphological identification of *P. amphibia* voucher 2012PolAm_Nevrokopi

Morphology. It is a rhizomatous perennial herb that reaches a height of 30 cm to 40 cm (**Fig. 1A,B**). It appears in late spring until mid autumn.

Description. Rhizomes: pencil-thick rhizomes (**Fig. 1C**). **Stems:** Most of the stems are branched at the base, erect or procumbent, hairless or appressed-haired. **Internodes:** 3 cm to 4 cm in length. **Ochrea:** hairy; 10 mm to 20 mm long. **Leaves:** simple with entire leaf blade edge; dull green; lanceolate; leaf blade length/width = (12–15) cm/(2–3) cm; rounded to cordate at base; acute apex; some plants (<5 %) appear with dark chevron-shaped blotches on leaves; joined to above middle of ochrea (**Fig. 1D,E**). **Petioles:** very short, giving the impression that the leaf blade is directly attached to the ochrea. **Inflorescences:** usually two terminal, erect, cylindrical, short-spiked, 4 cm to 7 cm long, densely flowered racemes (**Fig. 1A**). **Flowers:** unisexual, pistillate, five tepals with bright red to pink colour (**Fig. 1A**). **Flowering period:** July to August.

Ecology: The climate of Kato Nevrokopi plateau is characterized as continental with cold winter and cool summer. The existence of this weed in that area is speculated to be due to a lake that, in the past, covered all the area. Possibly this weed emerged in its aquatic form due to the water. The subsequent loss of the water and lake disappearance presumably caused the transformation to the present, terrestrial, form of the weed. The soil of the area is characterized as light with good drainage and acidic (the majority of the soils have pH around 6). *P. amphibia* grows well in various types of soil such as sand, clays, loam, peat, manure, underwater silt and black mud (35), with pH between 3 to 8 (14). The plants usually emerge in the beginning of May, before or at the same time that potato emerges (**Fig. 1B**, **Fig. 2A**). The latter is planted by the end of April. *P. amphibia* has a rapid vegetative spread and grows best under full sunlight or only light shade, and heavy rainfall, high humidity and water-logging enhance rapid growth as well (35). Similar weather conditions with heavy rainfall and high humidity usually occur during the potato cultivation period, which favors the rapid growth of the weed in the area of Kato Nevrokopi (**Table 1**). Sculthorpe (39) reported rapid underground lateral spread in fertile terrestrial situations. Conditions of full sunlight in relatively nutrient-rich water or well-irrigated deep soil, conditions that occur in potato fields, support and favor the growth of *P. amphibia* (35).

P. amphibia has become one of the most troublesome weed species in potato crops in Kato Nevrokopi (**Fig. 2A,B**), not only due to the favorable soil and weather conditions, but also due to the vegetative reproduction, the inefficient chemical control and the potato monoculture. Grime et al. (14) described the potential of a single 2 cm section of rhizome with a node to start a new colony. The weed is also dispersed and simultaneously propagated during ploughing, owing to stem and rhizome fragments, which can spread laterally more than 50 mm a day (39). This weed is considered as 'difficult to control and persisting' (15) and has become a serious problem in potato crop fields in the area of Kato Nevrokopi. The efficient control of other weed species in potato increases the growth and development of the uncontrolled *P. amphibia* (**Fig. 2B**). The incessant potato monoculture in this area, the lack of crop rotation and the application of the same methods of weed control have also made *P. amphibia* a widespread weed species difficult to control.

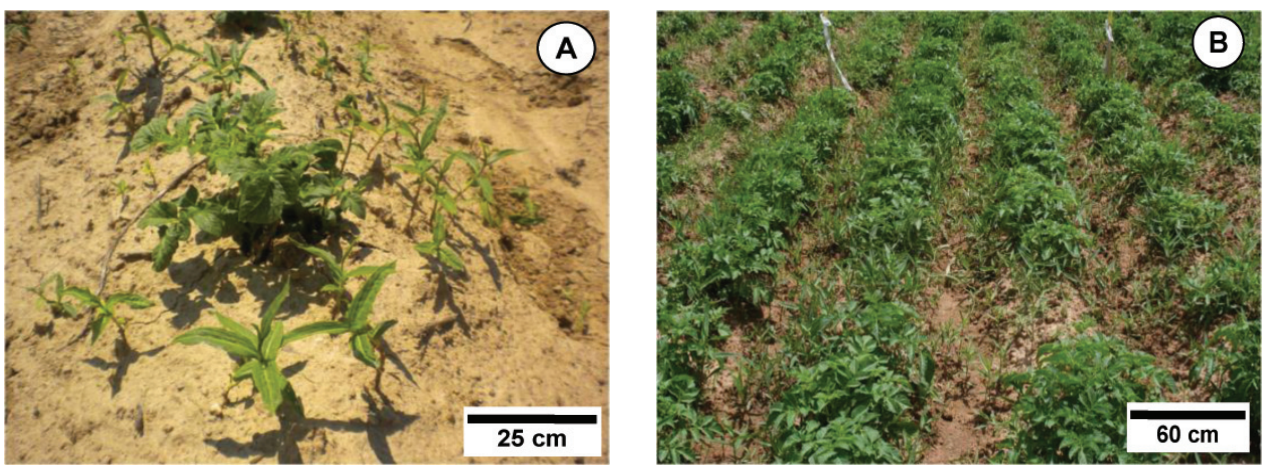

Fig. 2. Uncontrolled *Persicaria amphibia* voucher 2012PolAm_Nevrokopi plants grown in a potato field in Kato Nevrokopi, Greece: early infestation (**A**) and high infestation (**B**).

TABLE 1

Annual summary of temperature and precipitation in Kato Nevrokopi, Greece in 2012

Month	Mean Temperature (°C)	Mean min temperature (°C)	Mean max temperature (°C)	Precipitation (mm)
1	-5.6	-10.5	-0.2	79.3
2	-2.0	-6.7	3.2	89.6
3	5.4	-1.2	13.2	21.2
4	11.6	4.9	18.4	61.4
5	14.7	8.6	21.8	147.2
6	21.2	13.0	29.7	4.4
7	23.7	14.9	33.5	58.6
8	21.7	13.2	31.6	65.2
9	18.0	10.2	27.2	74.6
10	13.3	6.6	22.0	35.0
11	7.6	3.2	13.8	65.2
12	-0.1	-4.0	4.8	130.6

Source: *penteli.meteo.gr/stations/nevrokopi/NOAAPRYR.TXT*

TABLE 2

Molecular identification of *Persicaria amphibia* voucher 2012PolAm_Nevrokopi based on six molecular markers of cp DNA

Region	***matK***	***rbcL***	***rpoB***	***rpoC1***	***atpF-atpH***	***psbK-psbI***
Aligned sequence length (bp) of specimen	1196	596	490	504	603	452
	Nucleotide sequence blast and identification in percentage					
Persicaria amphibia	721/721, 100 %	570/570, 100 %				
Polygonum amphibium voucher Kim 600		565/565, 100 %				
Polygonum amphibium voucher Kim & Kim Ch-Ko-91		564/565, 99.8 %				
Persicaria maculosa	780/799, 98 %	577/587, 98 %	467/472, 99 %	482/485, 99 %	577/608, 95 %	397/421, 94 %
Persicaria hydropiper	761/778, 98 %	583/596, 98 %	466/472, 99 %	501/504, 99 %	574/610, 94 %	399/427, 93 %
Polygonum chinense	641/692, 93 %	580/593, 98 %	453/471, 96 %	469/484, 97 %		
Polygonum bistorta	893/984, 91 %		450/471, 96 %	469/482, 97 %		
Polygonum aviculare			451/470, 96 %	473/494, 96 %	548/622, 88 %	342/449, 83 %
Polygonum orientale			467/472, 99 %	481/485, 99 %		
Persicaria tinctoria			466/472, 99 %	481/485, 99 %		
Persicaria senticosum		592/596, 99 %				
Persicaria virginiana	244/251, 97 %	577/583, 99 %				
Persicaria filiformis	129/133, 97 %	572/576, 99 %				
Persicaria lapathifolia	725/740, 98 %			482/485, 99 %		

Sequence accession numbers obtained from GenBank and used in the present study:
Persicaria amphibia, AY395553, JN895115, *Polygonum amphibium* voucher Kim 600, EF653776, *Polygonum amphibium* voucher Kim & Kim Ch-Ko-91, EF653777, *Persicaria maculosa*, EU749114, EU676952, EU749345, EU750256, EU749561, EU749804, *Polygonum orientale*, EU554030, FJ503023, *Persicaria hydropiper*, EU749112, AB008781, EU749342, HQ594084, EU749558, *Persicaria tinctoria*, EU554040, FJ503033, *Polygonum chinense*, EU554037, EU554020, FJ503030, GQ434134, *Polygonum bistorta*, EU554028, AF204859, FJ503021, *Polygonum aviculare*, EU554029, FJ395748, FJ395265, *Persicaria senticosum*, D86289, *Persicaria virginiana*, FM883641, EF438019, *Persicaria filiformis*, AB125344, EF653722, *Persicaria lapathifolia*, HM357922, FJ503032, *Persicaria capitata,* FM883623, HM851074, *Persicaria runcinata,* AF297124, AY042627
New sequences generated from this study: *Persicaria amphibia*, KC342450, KC342451, KC342452, KC342453, KC342454 and KC342455

Molecular identification of *P. amphibia* voucher 2012PolAm_Nevrokopi

A total of 3841 nucleotides of six deferent cpDNA regions were sequenced for the specimen of *P. amphibia* voucher 2012PolAm_Nevrokopi, which causes severe losses during potato cultivation in Northern Greece. The sequences generated for this specimen have been deposited in GenBank under the following accession numbers: KC342450 for the *rpoB* gene, KC342451 for the *rbcL* gene, KC342452 for the *rpoC1* gene, KC342453 for the *matK* cpDNA region, KC342454 *atpF-atpH* region and KC342455 for the *psbK-psbI* region.

Alignment of these sequences to GenBank entries blasted only 93.86 % (3605/3841) identity with various *Persicaria* species. The specimen was identified as *P. amphibia*, with 100 % identity, based on the sequences with accession numbers AY395553, EF653776, EF653777 and JN895115, covering 33.61 % (1291/3841) of the total nucleotides sequenced for this specimen. The remaining sequences were aligned and demonstrated less than 100 % identity to other *Persicaria* species (**Table 2**). Therefore, a total of 2550 new nucleotides located in four different cpDNA regions of *psbK-psbI*, *atpF-atpH*, *rpoC1* and *rpoB* are now available at GenBank for *P. amphibia*.

The aligned sequence of the *rbcL* gene (KC342451) consists of 596 nucleotides which were identified as *P. amphibia* based on 100 % nucleotide similarity with *P. amphibia* (AY395553), (40), *P. amphibium* voucher Kim 600 (EF653776) and 99.8 % similarity with only one nucleotide difference (564/565) with *P. amphibium* voucher Kim & Kim Ch-Ko-91 (EF653777), (22), (**Table 2**). A phylogenetic dendrogram (**Fig. 3**) shows the genetic distance with eight other different *Persicaria* species, based on nucleotide differences at 27 loci out of 565 within the *rbcL* gene plastid region. These results confirm the general statement made by the CBOL (4) that *rbcL* offers high universality and good, but not outstanding, discriminating power.

The sequence of *matK* cpDNA region (KC342453) consists of 1196 nucleotides encoding two partial sequences of the *trnK* and *matK* genes. The sequence alignment of the specimen with *P. amphibia* (AY395553) resulted in 100 % (570/570) identity and there are now additional 626 new nucleotides available at GenBank for the *P. amphibia matK* gene region. A phylogenetic dendrogram (**Fig. 4**) shows the genetic distance with nine other different *Persicaria* species, based on nucleotide differences at 100 loci out of 701 within the *matK* gene plastid region. The data for the *matK* gene confirm the assumption made by Lahaye et al. (27) that *matK* alone should constitute a universal plant DNA barcode. However, the 2-locus barcode combinations, *rbcL_matK* gave better results for *P. amphibia* identification.

The next four molecular DNA barcodes investigated here provide new information for *P. amphibia* in GenBank and would, therefore, be useful for future research, particularly for *P. amphibia*. The aligned sequence of the *rpoB* gene (KC342450) consists of 490 nucleotides and was found to have 99 % similarity with *P. maculosa*, *P. hydropiper*, *P. orientalis* and *P. tinctoria*. The sequence of the *rpoC1* gene (KC342452) consists of 504 nucleotides and was found to have 99 % similarity with *P. hydropiper*, *P. orientalis* and *P. tinctoria*. The sequence of the *psbK-psbI* plastid region (KC342455) consists of 452 nucleotides and was found to have 94 % similarity with *P. maculosa*, 93 % with *P. hydropiper* and 83% with *P. aviculare*. The sequence of the *atpF-atpH* plastid region (KC342454) consists of 603 nucleotides and was found to have 95 % similarity with *P. maculosa*, 94 % with *P. hydropiper* and 88 % with *P. aviculare*.

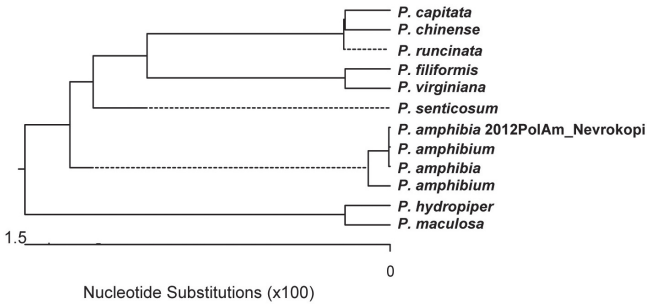

Fig. 3. Strict consensus tree illustrating the phylogeny of the new weed *P. amphibia* in Greece and related species, based on *rbcL* gene region and generated by the ClustalW method using the equations described by Thompson et al. (42). The dendrogram shows the identity of *P. amphibia* voucher 2012PolAm_Nevrokopi, with three other *P. amphibia* samples (EF653776, EF653777 and AY395553).

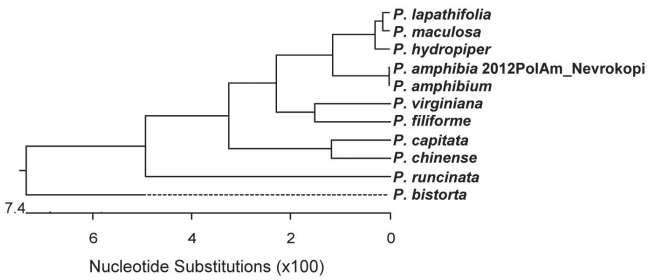

Fig. 4. Strict consensus tree illustrating the phylogeny of the new weed *P. amphibia* in Greece and related species, based on *matK* gene region and generated by the ClustalW method using the equations described by Thompson et al. (42). The dendrogram shows the identity of *Persicaria amphibia* voucher 2012PolAm_Nevrokopi, with one *P. amphibia* sample (JN895115).

These cpDNA regions have been partially investigated for phylogenetic studies of 14 different *Persicaria* species for *matK* region, 35 for the *rbcL* gene, nine for the *rpoB* gene, nine for the *rpoC1* gene, three for the *psbK-psbI* region and three for the *atpF-atpH* region, showing distinct nucleotide differences (10, 17, 22, 29). The generated sequences of the *rpoB* and *rpoC1* genes, as well as the *psbK-psbI* and *atpF-atpH* plastid regions are now new entries for *P. amphibia* at GenBank.

Conclusions

The results of this study suggest that the ideal DNA barcodes for the maximal discrimination of *Persicaria* species focus on *psbK-psbI* and *atpF-atpH* plastid regions. These chloroplast

regions are also recommended by the CBOL Plant Working Group (4), as universal barcodes to identify specimens of land plants and have only been applied for *P. maculosa*, *P. hydropiper* and *P. aviculare*. New sequence information for *psbK-psbI*, *atpF-atpH*, *rpoC1* and *rpoB* (a total of 2550 bp) is now available at GenBank and these four new cpDNA barcodes could be used for molecular classification of other *P. amphibia* specimens. The best molecular marker for the identification of *P. amphibia* was the *matK* gene, showing nucleotide differences at 100 loci out of 701, based on the results of the six plastid regions investigated in the present study. The rapid molecular classification of *Persicaria* specimens is a valuable genetic tool to identify current and future infestations caused by *Persicaria* species or subspecies in different crops and locations worldwide. Both molecular and morphological data (mainly the fact that leaves are joined to above the middle of the ochrea) provide useful information for the identification and taxonomy of the terrestrial form of this weed species recorded for the first time in Greece.

REFERENCES

1. **Altschul S.F., Madden T.L., Schaffer A.A., Zhang J., et al.** (1997) Nucleic Acids Res, **25**, 3389-3402.
2. **Brandbyge J.** (1993) In: The Families and Genera of Vascular Plants. II. Flowering Plants (Dicotyledons): Magnoliid, Hamamelid and Caryophyllid families (K. Kubitzki, J.G. Rohwer, V. Bittrich, Eds.), Springer-Verlag, Berlin, 531-544.
3. **Burke J.M., Sanchez A., Kron K., Luckow M.** (2010) Am. J. Bot., **97**, 1377-1390.
4. **CBOL Plant Working Group** (2009) P. Natl. Acad. Sci. USA, **106**, 12794-12797.
5. **Chase M.W., Cowan R.S., Hollingsworth P.M., van den Berg C., et al.** (2007) Taxon, **56**, 295-299.
6. **Decraene L.-P.R., Akeroyd J.R.** (1988) Bot. J. Linn. Soc., **98**, 321-371.
7. **Devey D.S., Chase M.W., Clarkson J.J.** (2009) Taxon, **58**, 7-15.
8. **Ekman S., Knutsson, T.** (1994) Nord. J. Bot., **14**, 23-25.
9. **Fassett N.C**. (1949) Brittonia, **6**, 369-393.
10. **Fazekas A.J., Burgess K.S., Kesanakurti P.R., Graham S.W., et al**. (2008) PLoS ONE, **3**, e2802.
11. **Ford C.S., Ayres K.L., Haider N., Toomey N., et al.** (2009) Bot. J. Linn. Soc., **159**, 1-11.
12. **Galasso G., Banfi E., Mattia F.d., Grassi F., et al.** (2009) Atti della Societ à Italiana di Scienze Naturali e del Museo Civico di Storia Naturale di Milano, **150**, 113-148.
13. **Greene E.L**. (1904) Leaflets of Botanical Observation and Criticism, Washington DC, pp. 17-32.
14. **Grime, J.P., Hodgson, J.G., Hunt R.** (1988) Comparative Plant Ecology: a Functional Approach to Communities of British Species, Unwin Hyman Publ., London.
15. **Hanf M.** (1983) The Arable Weeds of Europe, with their Seedlings and Seeds, BASF United Kingdom, p. 400.
16. **Haraldson K**. (1978) Symbolae Botanicae Upsalienses, **22**.
17. **Hilu K.W., Black C., Diouf D, Burleigh J.G.** (2008) Mol. Phylogenet. Evol., **48**, 1120-1130.
18. **Hollingsworth P.M., Graham S.W., Little D.P.** (2011) PLoS ONE, **6**, e19254.
19. **Jones J.** (2001) Laboratory Guide for Conducting Soil Tests and Plant Analysis, CRC Press, Boca Raton FL.
20. **Kent D.H**. (1992) List of Vascular Plants of the British Isles, Botanical Society of the British Isles, London.
21. **Kim J.S., Hong J.-K., Chase M.W., Fay M.F., et al.** (2013) Bot. J. Linn. Soc., **172**, 5-21.
22. **Kim S.-T., Donoghue M.J.** (2008). Syst. Bot., **33**, 77-86.
23. **Kim S.-T., Sultan S.E., Donoghue M.J.** (2008) P. Natl. Acad. Sci. USA, **105**, 12370-12375.
24. **Kress W.J., Erickson D.L.** (2007) PLoS ONE, **2**, e508.
25. **Kress W.J., Wurdack K.J., Zimmer E.A., Weigt L.A., et al.** (2005) P. Natl. Acad. Sci. USA, **102**, 8369-8374.
26. **Kwak M., Kim M.-H., Won H, Park C.-W.** (2006) Korean Journal of Plant Taxonomy, **36**, 21-39.
27. **Lahaye R., van der Bank M., Bogarin D., Warner J., et al.** (2008) In: Proceedings of the National Academy of Sciences of USA, **105**, 2923-2928.
28. **Li A.J., Li A.-R., Bojian B., Grabovskaya-Borodina A.E., et al.** (2003) Polygonaceae. Flora of China 5. Science Press, Beijing and Missouri Botanical Garden Press, St. Louis, pp. 277-350
29. **Li D.Z., Gao L.M., Li H.T., Wang H., et al.** (2011) P. Natl. Acad. Sci. USA, **108**, 19641-19646.
30. **Lousley J.E., Kent D.E.** (1981) Docks and Knotweeds of British Isles, Botanical Society of the Bitish Isles, London.
31. **Mitchell R.S., Dean J.K.** (1978) N. Y. State Mus. Bull., **431**, 1-79.
32. **Mitchell R.S.** (1968) University of California Publications in Botany, **45**, 1-54.
33. **Newmaster, S.G., Ragupathy, S., Janovec J.** (2009) Int. J. Comp. Appl. Tech., **35**, 50-60.
34. **Olmstead R.G., Palmer J.D**. (1994) Am. J. Bot., **81**, 1205-1224.
35. **Partridge W.J.** (2001) J. Ecol., **89**, 487-501.
36. **Rai I.D., Singh G., Rawat G.S.** (2013) Phytotaxa, **92**, 13-19.
37. **Rodwell J.S.** (1995) British Plant Communities, Vol. 4, Aquatic Communities, Swamps and Tall-Herb Fens, Cambridge University Press, Cambridge.
38. **Sanchez I., Kron K.A**. (2008) Syst. Bot., **33,** 87-96.
39. **Sculthorpe C.D.** (1967) The Biology of Aquatic Vascular Plants, Edward Arnold, London.
40. **Silvertown J., McConway K., Gowing D., Dodd M., et al.** (2006) P. Roy. Soc. B-Biol. Sci., **273**, 39-44.
41. **Stace C.A.** (1997) New Flora of the British Isles, 2nd Ed., Cambridge University Press, Cambridge.
42. **Thompson J.D., Higgins D.G., Gibson T.J.** (1994) Nucleic Acids Res., **22**, 4673-4680.
43. **Van der Weide R.Y., Bleeker P., Riemens M., Bastiaans L.** (2008) In: EWRS-NJF Workshop, 10-12 November 2008, Wageningen, p. 5.
44. **Zhao D.-P., Wang K.-M., Hou Y.-T.** (2012) Bulletin of Botanical Research, **32**, 77-83.