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A multi-marker DNA barcoding approach to save time and resources in vegetation surveys

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Vegetation surveys have a long tradition in ecological studies, but several limitations in the morphological identification of species have been recognized. The objective of this study was to evaluate the effectiveness of DNA barcoding in plant species identification to save field technicians time and resources. Vegetation surveys were performed in four plots of semi-dry grassland in the Italian subalpine region of Lombardy. Two identification approaches were employed: a conventional morphological identification and a molecular multi-marker DNA barcoding method. Results showed that morphological identification of 49 species collected from the study area (five field inspections) required a substantial amount of time to complete relative to the molecular method. The same 49 samples were analysed using the following DNA multi-marker barcodes: *rbcL*, *matK* and *trnH-psbA*. *rbcL* showed 100% amplification success with standard primers, but low interspecific genetic variability. *matK* demonstrated some amplification problems with standard primers; however, consistent genetic diversity was observed. Finally, the *trnH-psbA* spacer region exhibited reliable amplification success and the highest molecular variability. In a comparison with publicly available databases, *trnH-psbA* and *matK* returned the highest proportion of identified samples, whereas *rbcL* returned several misidentifications. The DNA barcoding approach is a powerful tool in vegetation surveys and may significantly reduce the time and cost spent for species identification. However, to effectively apply DNA barcoding in vegetation surveys, exhaustive local or regional molecular databases must be defined. *Botanical Journal of the Linnean Society*, 2012, **169**, 518–529.

ADDITIONAL KEYWORDS: integrated taxonomy – *matK* – plastid DNA markers – *rbcL* – *trnH-psbA* – vegetation plot.

INTRODUCTION

Vegetation survey techniques based on plot sampling have a long tradition in Europe and North America, dating to the early 20th century. Today, the approach is primarily used to evaluate plant biodiversity at local or regional scales and to monitor the effects of natural or anthropogenic environmental pressures (Gentili *et al*., 2010; Bordenave, De Granville & Steyn, 2011). The method involves the characterization of the vegetation cover, species composition and proportion of different taxa associated with natural communities (Mueller-Dombois & Ellenberg, 1974). Among the classical plant association-based approaches (Clements, 1905), the phytosociological method has become the standard for many vegetation scientists to classify and rank plant communities (Westhoff & van der Maarel, 1973; Dengler, Chytry & Ewald, 2008).

Researchers face two primary challenges when conducting vegetation surveys, resulting in time and cost constraints: field data collection and subsequent data processing. Currently, digital storage and data analysis procedures have become increasingly more

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manageable and less complex than in the past as a result of the development of specialized software (Mucina & van der Maarel, 1989). However, field work is the principal time-consuming activity, and plant identification often requires the survey of many vegetation parameters (e.g. species association details, ecological parameters) and the collection of voucher specimens, which requires species to be definitively identified (Alados *et al*., 2006). Species identification is usually accomplished by means of dichotomous keys, with additional confirmation, if necessary, by morphological comparisons with herbarium reference specimens or working with botanists experienced in different plant groups. Indeed, plant identification requires experience, technical skills and knowledge of local flora, including new and exotic species (Funk, Richardson & Ferrier, 2005; Newmaster, Ragupathy & Janovec, 2009).

Molecular approaches can overcome excessive costs during vegetation data collection. Among the range of available techniques, 'DNA barcoding' is currently one of the most economic and reliable (Hebert *et al*., 2003; http://www.barcodeoflife.org). One or more standard DNA regions of the plant genome are amplified and sequenced using a small tissue sample (e.g. vegetative material, fruit or flower). The barcode sequences generated are compared with a reference sequence library, and a sequence similarity match with sequences included in a reference dataset results in rapid and reproducible taxonomic recognition (Hebert *et al*., 2003; Hollingsworth, 2007; Bruni *et al*., 2010). The Consortium for the Barcode of Life (CBOL) Plant Working Group proposed the plastid gene *matK* and *rbcL* regions (plastid DNA) as universal barcodes for plants (Hollingsworth *et al*., 2009). In addition, the plastid intergenic spacer region *trnH-psbA* has been recommended recently (Fazekas *et al*., 2010b; Hollingsworth, Graham & Little, 2011). Although many questions remain open with regard to the most suitable DNA region(s) for plant DNA barcoding (Chase *et al*., 2007; Hollingsworth, 2008; Ford *et al*., 2009; Fazekas *et al*., 2010b), and a plant reference sequence library has not yet been defined, DNA barcoding has allowed taxonomists to embark on new avenues of study in plant systematics.

In this work, we evaluated the efficacy of different DNA barcode markers (*matK*, *rbcL* and *trnH-psbA*). Our tests were conducted in the Italian subalpine region of Lombardy, using a vegetation plot survey as a model. The study area is a mosaic of open semi-dry grassland vegetation, with shrubs and tree species colonizing and covering areas of the plant community (Fig. 1). The European Habitats Directive 92/43/EEC classifies the community type as Festuco-Brometalia grasslands (Habitat 6210), which occurs on calcareous substrate. The aim of the present study was to test the congruence between the application of classical floristic methods and DNA barcoding in plant species

Figure 1. Panoramic view of the study area (Italian pre-alpine region). Photographs showing the main characteristics of the study area in which the vegetation survey was conducted. The left-hand figure shows the semi-dry grassland (open plots were selected in this subarea) surrounded by shrubs and trees, such as *Corylus avellana* L. (below) and *Fagus sylvatica* L. (above). Where shrub and tree species tended to colonize and cover the open grassland, we identified the closed plots. The right-hand figure shows the details of grassland with typical semi-dry vegetation and the high slope incline (open plots).

identification, and the capacity of each method to assess levels of diagnostic variation. The results of our analyses allowed us to emphasize the benefits and limitations of DNA barcoding in vegetation surveys. Furthermore, we evaluated the utility of the method to assist in special status species (threatened, rare or endangered) management plans or conservation guidelines.

MATERIAL AND METHODS

Vegetation plot analysis

Vegetation plot analysis was conducted in the Italian pre-alpine region (Lombardy, Valcuvia, VA: 45°55′N; 08°39–40′E). Four equally sized vegetation plots (3×3 m^2) were surveyed: two in open grassland and two in closed grassland, where *Corylus avellana* L. tends to colonize and cover a great part of the surface. Vegetation relief and sampling were conducted from April to August 2009 with a total of five inspections (once a month).

The ground cover was estimated for each species in each plot using percentage cover abundance values. The following parameters were also measured for each species: (1) number of individuals, clumps or flowering stems; (Ni) (2) maximum canopy size (Lm); (3) maximum height (Hm). The identification of each species was performed both morphologically and with molecular tools, as described below.

Morphological identification of plants

During each site inspection, three to five samples belonging to all the species distributed within each plot were collected for morphological identification. All experiments, procedures and ethical issues were in conformity with the competent national ethical bodies; in particular, sampling activities were conducted according to Permesso di Campionamento N° DPN/2D/2004/13650 granted by the Ministero dell'Ambiente della Repubblica Italiana. Morphological identification was conducted in collaboration with local taxonomists and based on dichotomous keys (Pignatti, 1982; Aeschimann *et al*., 2004; Macchi, 2005). Direct comparisons were accomplished with reference to type specimens archived in the herbarium of the Natural History Museum of Milan, Italy (MSNM). One individual for each collected species was deposited in the same herbarium.

DNA barcoding analyses

All of the plant samples were collected in two field inspections in June and August, respectively. Two or three young leaves were collected for each species, placed in Eppendorf tubes stored in ice and transferred to a –20 °C refrigerator. Samples were vouchered as 'MIB:ZPL' following the protocols specified by the biorepositories initiative (http://www.biorepositories.org) and the data standards for BARCODE Records in the International Nucleotide Sequence Database Collaboration (INSDC) (http://barcoding.si.edu/PDF/DWG_data_standards-Final.pdf). A list of samples and voucher codes is included in Table 1. A total of 100 mg of plant material was used for DNA extraction. Genomic DNA was isolated using the DNeasy Isolation and Purification kit (Qiagen, Milan, Italy), allowing high-quality DNA, free of polysaccharides or other metabolites that might interfere with DNA amplification, to be obtained (Bruni *et al*., 2010).

Molecular characterization was performed with three different DNA markers widely used in a DNA barcoding context (Hollingsworth *et al*., 2009; Bruni *et al*., 2010): two coding (*rbcL* and *matK*) and one noncoding (*trnH-psbA* intergenic spacer) plastid DNA region. Polymerase chain reaction (PCR) amplification was performed using puReTaq Ready-To-Go PCR beads (Amersham Bioscience, Italy) in a 25-μL reaction according to the manufacturer's instructions. PCR cycles consisted of an initial denaturation step for 7 min at 94 °C, 35 cycles of denaturation (45 s at 94 °C), annealing (30 s at different temperatures; see Table 2) and extension (1 min at 72 °C), and a final extension at 72 °C for 7 min. Further details on primer pairs and amplification conditions are provided in Table 2. PCR products were bidirectionally sequenced using an ABI 155 3730XL automated sequencer at Macrogen Inc., South Korea. Manual editing of raw traces and subsequent alignments of forward and reverse sequences allowed us to assign edited sequences for most species. The 3′ and 5′ terminals were clipped to generate consensus sequences for each taxon. In order to avoid the inclusion of inadvertently amplified nuclear pseudogenes of plastid origin (see, for example, Naciri & Manen, 2010), barcode sequences were checked following the guidelines proposed in Song *et al*. (2008) and Buhay (2009). Finally, the sequences obtained were deposited in the EMBL Data Library.

Data analysis

The first step of the work was to provide evidence for the universality of the three candidate DNA barcodes. For this reason, we evaluated which DNA markers were routinely amplified and sequenced in the highest number of analysed samples. Only the most universal primer combinations for each candidate marker were tested (Table 2). For all taxa and loci, we conducted PCR amplification in a two-stage trial. In the first stage, we used the standard PCR conditions described above, starting from 10 ng of DNA tem-

Table 1. Species composition of the four vegetation plots in the semi-arid grassland of Valcuvia (Italian pre-alps)

Code	Species	Life form	Phenology (Pignatti, 1982) M	AP	MA	JN	JL	AG	SP	OC	Determination	Plot 1 closed Cover (%)	N_i	H_m	L_m
MIB:Zpl:02034	*Allium sphaerocephalon* L.	G									Jl				
MIB:Zpl:02799	*Anthericum liliago* L.	G									J				
MIB:Zpl:03832	*Aquilegia atrata* Koch	H									J	0.5	1	4	2
MIB:Zpl:02018	*Arabis hirsuta* (L.) Scop.	H									J				
MIB:Zpl:01703	*Asperula purpurea* (L.) Ehrend.	CH									A	1	14	20	5
MIB:Zpl:02029	*Aster amellus* L.	H									A				
MIB:Zpl:02021	*Brachypodium rupestre* (Host) Roem. & Schult.	H									J	3	28	61	10
MIB:Zpl:02035	*Briza media* L.	H									Jl				
MIB:Zpl:02014	*Bromus erectus* Hudson	H									J	1	5	73	5
MIB:Zpl:03840	*Calamagrostis arundinacea* (L.) Roth	H									Jl				
MIB:Zpl:02019	*Campanula rotundifolia* L.	H									A				
MIB:Zpl:01686	*Carex humilis* Leyser	H									Mj	60	55	35	20
MIB:Zpl:02808	*Carlina acaulis* L.	H									Jl				
MIB:Zpl:01705	*Centaurea bracteata* Scop.	H									Jl				
MIB:Zpl:02807	*Chrysopogon gryllus* (L.) Trin.	H									Jl				
MIB:Zpl:02812	*Clematis vitalba* L.	P									J	10	2	5	300
MIB:zpl:01701	*Colchicum autumnale* L.	G									Jl	1	8	37	6
MIB:Zpl:02022	*Coronilla emerus* L.	NP									A				
MIB:Zpl:02028	*Corylus avellana* L.	P									Ap	90	2	350	180
MIB:Zpl:01692	*Dactylis glomerata* L.	H									J	0.5	6	62	5
MIB:Zpl:01714	*Dorycnium pentaphyllum* Scop.	H									J				
MIB:Zpl:02801	*Erica carnea* L.	CH									Mj				
MIB:Zpl:02802	*Fumana procumbens* (Dunal) Gren. & Godr.	CH									J				
MIB:Zpl:02810	*Galium lucidum* All.	H									Jl				
MIB:Zpl:01683	*Geranium sanguineum* L.	T									Jl	0.5	1	16	3.5
MIB:Zpl:01709	*Globularia cordifolia* L.	H									Mj				
MIB:Zpl:02032	*Helianthemum nummularium* (L.) Miller	CH									Jl				
MIB:Zpl:02016	*Hieracium murorum* L.	CH									J				
MIB:Zpl:03837	*Hippocrepis comosa* L.	H									Jl				
MIB:Zpl:01671	*Inula hirta* L.	H									A				
MIB:Zpl:01672	*Inula salicina* L.	H									Jl				
MIB:Zpl:03839	*Juniperus communis* L.	P									Ap				
MIB:Zpl:02803	*Leontodon hispidus* L.	H									A				
MIB:Zpl:03979	*Mercurialis perennis* L.	G									J	0.5	3	24	9
MIB:Zpl:01721	*Origanum vulgare* L.	H									Jl				
MIB:Zpl:02013	*Pimpinella saxifraga* L.	H									Jl				
MIB:Zpl:02814	*Polygala chamaebuxus* L.	CH									J	0.5	7	8	4
MIB:Zpl:02027	*Prunella grandiflora* (L.) Scholler	H									J				
MIB:Zpl:03316	*Prunus spinosa* L.	P									Ap	0.5	1	25	11
MIB:Zpl:02811	*Pteridium aquilinum* (L.) Kuhn	G									Jl	10	7	145	87
MIB:Zpl:02033	*Rubus fruticosus* aggr.	NP									J				
MIB:Zpl:03525	*Sanguisorba minor* Scop.	H									A				
MIB:Zpl:02017	*Sesleria varia* (Jacq.) Wettst.	H									Jl	0.5	1	46	2
MIB:Zpl:03205	*Tamus communis* L.	H									Mj	0.5	15	6	5
MIB:Zpl:03522	*Tanacetum corymbosum* (L.) Sch.-Bip.	G									Jl	0.5	2	12	5
MIB:Zpl:01718	*Teucrium chamaedrys* L.	H									J	0.5	26	12	5
MIB:Zpl:01702	*Teucrium montanum* L.	CH									Jl				
MIB:Zpl:02012	*Viola hirta* L.	H									Ap	0.5	3	7	4
MIB:Zpl:02020	*Viola reichenbachiana* Jordan ex Boreau	H									Mj				
			MAR	AP	MA	JN	JL	AG	SP	OC		9.552632	9.84	49.9	35.2
			9	13	28	40	35	24	11	5					Ni
												Mean closed			7.72
												SD			3.0

Code	Species	Plot 2 closed				Plot 3 opened				Plot 4 opened			
		Cover (%)	N_i	H_m	L_m	Cover (%)	N_i	H_m	L_m	Cover (%)	N_i	H_m	L_m
MIB:Zpl:02034	*Allium sphaerocephalon* L.					0.5	3	50	2.5				
MIB:Zpl:02799	*Anthericum liliago* L.									0.5	5	42	12
MIB:Zpl:03832	*Aquilegia atrata* Koch												
MIB:Zpl:02018	*Arabis hirsuta* (L.) Scop.	1	7	25	11								
MIB:Zpl:01703	*Asperula purpurea* (L.) Ehrend.												
MIB:Zpl:02029	*Aster amellus* L.	0.5	5	20	7.5	1	15	15	8	0.5	7	15	4
MIB:Zpl:02021	*Brachypodium rupestre* (Host) Roem. & Schult.	15	0	37	4.5	0.5	0	41	7				
MIB:Zpl:02035	*Briza media* L.					0.5	3	46	3	0.5	1	48	9
MIB:Zpl:02014	*Bromus erectus* Hudson					1	16	67	2.5	0.5	1	60	3
MIB:Zpl:03840	*Calamagrostis arundinacea* (L.) Roth						0	25	3.5				
MIB:Zpl:02019	*Campanula rotundifolia* L.					0.5	30	24	5				
MIB:Zpl:01686	*Carex humilis* Leyser	20	40	25	3	65	90	21	10	20	60	20	2
MIB:Zpl:02808	*Carlina acaulis* L.									0.5	1	4	18
MIB:Zpl:01705	*Centaurea bracteata* Scop.					0.5	1	21	3				
MIB:Zpl:02807	*Chrysopogon gryllus* (L.) Trin.									0.5	1	130	30
MIB:Zpl:02812	*Clematis vitalba* L.	0.5	3	23	16								
MIB:zpl:01701	*Colchicum autumnale* L.												
MIB:Zpl:02022	*Coronilla emerus* L.	0.5	6	12	7								
MIB:Zpl:02028	*Corylus avellana* L.	95	0	400	300								
MIB:Zpl:01692	*Dactylis glomerata* L.												
MIB:Zpl:01714	*Dorycnium pentaphyllum* Scop.									0.5	44	19	2.5
MIB:Zpl:02801	*Erica carnea* L.					23	50	12	0.5	20	30	10	5
MIB:Zpl:02802	*Fumana procumbens* (Dunal) Gren. & Godr.									0.5	1	6	1.5
MIB:Zpl:02810	*Galium lucidum* All.	0.5	1	25	4	0.5	2	10	1.5				
MIB:Zpl:01683	*Geranium sanguineum* L.												
MIB:Zpl:01709	*Globularia cordifolia* L.					0.5	2	8	5	1	6	27	7
MIB:Zpl:02032	*Helianthemum nummularium* (L.) Miller					0.5	1	17	4	0.5	5	15	3
MIB:Zpl:02016	*Hieracium murorum* L.					0.5	2	3	7				
MIB:Zpl:03837	*Hippocrepis comosa* L.					0.5	7	12	3	0.5	6	7	3
MIB:Zpl:01671	*Inula hirta* L.	0.5	1	10	6								
MIB:Zpl:01672	*Inula salicina* L.					0.5	10	22	5				
MIB:Zpl:03839	*Juniperus communis* L.					0.5	1	36	10				
MIB:Zpl:02803	*Leontodon hispidus* L.					0.5	5	10	5				
MIB:Zpl:03979	*Mercurialis perennis* L.												
MIB:Zpl:01721	*Origanum vulgare* L.	0.5	4	16	5								
MIB:Zpl:02013	*Pimpinella saxifraga* L.	0.5	6	3	5								
MIB:Zpl:02814	*Polygala chamaebuxus* L.					1	18	6	3	0.5	10	5	1.5
MIB:Zpl:02027	*Prunella grandiflora* (L.) Scholler									0.5	18	12	6
MIB:Zpl:03316	*Prunus spinosa* L.												
MIB:Zpl:02811	*Pteridium aquilinum* (L.) Kuhn	2	10	121	77								
MIB:Zpl:02033	*Rubus fruticosus* aggr.	0.5	0	19	14								
MIB:Zpl:03525	*Sanguisorba minor* Scop.					0.5	6	12	2.5				
MIB:Zpl:02017	*Sesleria varia* (Jacq.) Wettst.					40	80	37	1.5	30	195	34	3
MIB:Zpl:03205	*Tamus communis* L.												
MIB:Zpl:03522	*Tanacetum corymbosum* (L.) Sch.-Bip.												
MIB:Zpl:01718	*Teucrium chamaedrys* L.	0.5	0	15	6.5	1	2	7	2.5	1	14	9.5	3
MIB:Zpl:01702	*Teucrium montanum* L.					3	95	10	2	0.5	50	10	3.5
MIB:Zpl:02012	*Viola hirta* L.	0.5	1	6	10	0.5	1	4	3				
MIB:Zpl:02020	*Viola reichenbachiana* Jordan ex Boreau	0.5	1	6	5								
		9.2	5.6	50.4	31.8	6.45455	19	20	4.24	4.36111	25.3	26.3	6.5
		Hm	Lm						Ni	Hm	Lm		
		24.7	13.7			Mean opened			22.1	22.6	4.7		
		0.4	2.4			SD			4.4	4.3	1.6		

Data and traits collected within each plot for each species include: voucher code of collected samples; species name; life form; phenology (according to Pignatti, 1982); optimum period (month) for a reliable morphological identification (Determination) in the study area. Moreover, for each plot, the percentage cover, number of individuals (i.e. number of rosettes or flowering stems, or stems: N_i), maximum height (H_m) and mean canopy (L_m) were provided. Highlighted areas (in grey) correspond to the flowering months under the column 'Phenology'.
AP, April; MA, May; JN, June; JL, July; AG, August; SP, September; OC, October; CH, chamaephyte; H, hemicryptophyte G, geophyte; N, nano-phanerophyte; P, phanerophyte; T, Terophyte.

Table 2. List of primer pairs and polymerase chain reaction (PCR) annealing temperatures used in the present study for the three selected DNA barcoding markers

Locus	Primer name	Sequences (5′–3′)	Annealing temperature (°C)	Reference
trnH-psbA	psbA	GTTATGCATGAACGTAATGCTC	53	Newmaster & Ragupathy (2009)
	trnH	CGCGCATGGTGGATTCACAATCC		
matK	matK 390	CGATCTATTCATTCAATATTC	48	Cuénoud *et al.* (2002)
	matK 1326	TCTAGCACACGAAAGTCGAAGT		
rbcL	rbcL 1F	ATGTCACCACAAACAGAAAC	48	Fay *et al.* (1998)
	rbcL 724R	TCGCATGTACCTGCAGTAGC		

plate. The PCRs of samples that did not amplify any fragment or that produced multiple or nonspecific PCR products were repeated at lower stringency conditions: reduction of 5 °C in the annealing temperature and 40 PCR cycles. In the case of a new failure, PCR products belonging to both stages were re-amplified using 1 and 20 ng of DNA template. Only in cases of negative amplification with all conditions was PCR considered to be a failure and the sample was removed from the dataset.

The performance of each marker was also evaluated by considering sequence length and alignment success. According to the guidelines provided by CBOL (http://www.barcoding.si.edu/protocols.html), the evaluation of comparative levels of variation and discrimination for the three markers were undertaken using MEGA 4.0 (Tamura *et al*., 2007) to generate Kimura two-parameter (K2P) distance matrices for each locus.

Finally, we simulated the identification of all samples via comparison with existing molecular databases. Although the CBOL Plant Working Group has initiated a plant DNA barcoding database based on *rbcL* and *matK* (see http://www.boldsystems.org), too few accessions have yet been deposited for a functional system, even for useful qualitative analyses. For this reason, we decided to exclude BOLD comparison results and used the BLAST search (Altschul *et al*., 1990) in GenBank. Identification results were provided as a list of the nearest matches (maximum identity) according to BOLD-IDS guidelines (http://www.boldsystems.org/views/idrequest_plants.php). The BLAST maximum identity matches were grouped into three categories: (1) 'identified' (ID), when the maximum identity scores corresponded to the queried species; (2) 'bad identification' (BI), when the BLAST search returned the same maximum identity score with more than one species that might correspond or not to that queried; (3) 'not identifiable' (NI), when the maximum identity scores were consistently below 100% and the correct species did not occur among the entries.

RESULTS

Morphological species identification and vegetation plot analyses

Five total field surveys were required to identify all 49 plant species distributed in the study area based on plant phenology and availability of the required morphologically diagnostic characters. The modest number of species detected in our field surveys was probably a result of the steep slope incline of the study area (Fig. 1) and the thin soil cover of the calcareous substrate, characteristic of the habitat type. Although the detected number of plant species was rather limited, it should again be noted that the EU recognizes this area as a priority habitat (6210) for high biodiversity and environmental protection because of the occurrence of rare and endangered species (e.g. *Aquilegia atrata* Koch.).

The vegetational analysis results are summarized in Table 1. A total of 15 and 18 species (approximately 31% and 37% of the total sampling, respectively) were unambiguously identified during the third and fourth inspections (i.e. June and July, respectively), corresponding to the peak flowering periods for most plants. However, four species (*Corylus avellana* L., *Juniperus communis* L., *Prunus spinosa* L. and *Viola hirta* L.) were identifiable during the first (April) survey. Eight species required microscopic analysis of morphological traits to reach a definitive identification, including *Arabis hirsuta* (L.) Scop., *Brachypodium rupestre* (Host) Roem. & Schult., *Calamagrostis arundinacea* (L.) Roth, *Chrysopogon gryllus* (L.) Trin., *Galium lucidum* All., *Leontodon hispidus* L., *V. hirta* and *V. reichenbachiana* Jordan ex Boreau.

Vegetation survey data showed that, of the 49 plant species detected, nine were common among all plots, 19 were exclusive to shrub-closed grassland plots and 21 to open grassland plots. The prevailing life forms were hemicryptophytes (H, 58%), chamaephytes (CH, 15%) and geophytes (G, 13%). Species with the highest mean percentage cover included *C. avellana*

(92.5%), *Carex humilis* Leyse (53.8%) and *Sesleria varia* (Jacq.) Wettst. (10.8%). Tree species dominated the shrub-closed grassland plots, and two herbaceous taxa (*C. humilis* and *S. varia*) were abundant in open grassland plots (Table 1). The mean number of species was 17.5 in shrub-closed plots and 21 in open plots. The mean numbers of individuals (N_i) in shrub-closed and open plots were 247 and 517, respectively, the mean maximum heights (H_m) in shrub-closed and open plots were 24.7 cm and 22.6 cm, respectively, and the mean canopy values (L_m) in shrub-closed and open plots were 13.7 cm and 4.7 cm, respectively.

The open and closed grassland plot comparisons suggested that, as a result of the absence of traditional activities, i.e. mowing and grazing, trees and shrubs have invaded herbaceous communities (e.g. *C. avellana*). These conditions have led to the progressive loss or fragmentation of grasslands and natural reforestation processes (Chemini & Rizzoli, 2003; Alados *et al*., 2006; Roura-Pascual *et al*., 2010), with a strong reduction in herbaceous species (see N_i values in Table 1).

DNA barcoding success

DNA extraction was successful for all 49 samples with high DNA quality and good yield (i.e. 30–50 ng μL^{-1}). Conspicuous differences in amplification success, PCR product lengths and sequence quality were detected for the three loci. In particular, amplification successes with standard primer pairs and thermal conditions were 100%, 90% (44 of 49 samples) and 57% (28 of 49 samples) for *rbcL*, *trnH-psbA* and *matK*, respectively. Although *matK* is considered to be one of the most suitable DNA barcodes for flowering plants because of its rapid evolution (see http://www.barcoding.si.edu/plant_working_group.html), our results were congruent with previous studies, which indicated the difficulties of working with a limited number of universal primer combinations to amplify this locus (Kress & Erickson, 2007; Fazekas *et al*., 2008; De Mattia *et al*., 2011). Recently, Dunning & Savolainen (2010) have defined an order-specific primer combination for monocots and eudicots, but the selection of suitable primer combinations for each plant sample required preliminary taxonomic identification (at least at the order or family level) before initiating the analyses. Compared with *matK*, *trnH-psbA* and *rbcL* typically required only one primer pair each for amplification, and both markers performed well. Even though, at the present state of the art, there are technical limits for the general use of *matK*, it is still a promising marker for plant DNA barcoding. As discussed recently at the Fourth International Barcode of Life Conference (http://www.dnabarcodes2011.org/), significant technical advances and an astounding amount of sequences will be made available by tremendous ongoing international projects.

PCR products were sequenced without complication, with the exception of one *rbcL* (i.e. *V. reichenbachiana* MIB:Zpl:02020), which resulted in a partial sequence of 473 bp. Accession numbers for each sequence obtained in the analysis are provided in Table S1 (see Supporting Information). Substantial sequence length differences were detected in the three markers as follows: 146–561 bp for *trnH-psbA*, 443–846 bp for *matK* and 473–610 bp for *rbc*L (data not shown).

Data expressed as the K2P molecular divergence, converted into percentages, indicated that *trnH-psbA* exhibited increased genetic diversity among all species, with a minimum of 1.50% between *Inula hirta* L. and *I. salicina* L. Genetic distance values were notably high among species of the same order [e.g. 57.5% between *Campanula rotundifolia* and *L. hispidus* (Asterales) and 56.2% between *Origanum vulgare* L. and *Teucrium montanum* L. (Lamiales)]. PCR priming sites in highly conserved flanking sequences, combined with a noncoding region possessing high substitution rates, made the *trnH-psbA* spacer highly suitable as a plant barcode. Previous research has reported the frequent occurrence of stutter PCR products for *trnH-psbA* as a result of mononucleotide repeats (Hollingsworth, 2008; Fazekas, Steeves & Newmaster, 2010a), but these technical issues were easily overcome using appropriate polymerases and PCR conditions (Fazekas *et al*., 2010a).

K2P for *matK* showed levels of variability ranging between 1.30% for *S. varia* and *Dactylis glomerata* L. and 25.7% for *S. varia* and *C. humilis*. *rbcL* showed the lowest genetic variability, with a minimum of 0% between *C. arundinacea* and *J. communis*, and a maximum of 25.4% between *Pteridium aquilinum* (L.) Kuhn and *C. rotundifolia*.

On the basis of these results, we conclude that *rbcL* is the most universal and the easiest to amplify, although it shows a moderate efficacy in the discrimination of different species. However, *trnH-psbA* is the most polymorphic and, consequently, the most suitable for the discrimination between closely related species (Newmaster *et al*., 2008).

Molecular species identification with BLAST

The barcode sequences were compared with the publicly available DNA barcode in GenBank to simulate a taxonomic assessment of our molecular data. In Table S2 (see Supporting Information), the first three BLAST maximum identity matches are reported. At December 2011, the GenBank database included sequences for 15 *trnH-psbA*, 22 *matK* and 24 *rbcL* for

the 49 species sampled in this study (Table 3). Among these, *trnH-psbA* and *matK* returned the most ID samples (13 of 15 and 17 of 22, respectively), whereas *rbc*L returned 16 of 24 cases of BI. Overall, in 21 of 49 cases, at least one marker correctly identified the query species.

We also decided to perform a BLAST search analysis for those sampled species for which any sequence was present in GenBank to evaluate the risk of misidentification. Our results based on all three barcode markers revealed the occurrence of NI in most cases (Table 3); however, some cases of BI were detected in both *trnH-psbA* and *rbcL* analyses.

DISCUSSION

The results of this study demonstrated that a multimarker DNA barcoding approach is a viable future tool for vegetation surveys. Indeed, if a robust reference library is available, integrative support for this methodology can substantially reduce the time and costs associated with field work. Table 4 shows the comparison of costs and benefits of classical vegetational analysis and DNA barcoding. Currently, the cost for a DNA barcoding analysis for a single plant sample with one marker is less than $3. Following a single DNA extraction, the cost for the analysis of *trnH-psbA*, *matK* and *rbcL* is approximately $7.55 (Table 4). It is difficult to generalize the absolute time spent in herbarium (preparation of samples, morphological identification) or molecular laboratory (data cleaning, BLASTing) analysis, because it relies on plant characteristics, operator ability and automation of the laboratory and data analysis pipelines. Recent studies (Newmaster *et al*., 2009; Fazekas *et al*., 2010a; Burgess *et al*., 2011) have clearly shown that, in molecular laboratory analyses, the costs decrease significantly with an increasing number of processed samples. Such a favourable situation does not occur in classical vegetation analyses, where each sample must be treated separately. Moreover, Newmaster & Ragupathy (2009) reported that the time and costs required for plant identification with traditional taxonomic methods were two-fold higher than those required using molecular approaches, consistent with the results of our study. On the whole, we suggest that, to complete the DNA barcoding field protocol, a limited amount of field time is required to collect and characterize plant biodiversity, which is independent of the specific life stage of the organism (Hollingsworth, 2007; Bruni *et al*., 2010; De Mattia *et al*., 2011). As a final consideration, we stress that vegetation surveys require experienced botanists specialized in the local flora to identify species on the basis of morphology, distribution and other diagnostic characters.

However, it is important to emphasize that DNA barcoding is only able to identify and determine the presence/absence of different species and cannot define morphological traits, age class, species cover and frequency, among other parameters. Another limitation of DNA barcoding is the use of uniparentally inherited plastid markers which cannot provide reliable identification of hybrids from parental species (Bruni *et al*., 2010; Naciri & Manen, 2010). The application of biparentally inherited nuclear markers might help to resolve this problem. However, several nuclear regions have been tested on plants with patchy results, and an ideal candidate is far from being characterized (Chase *et al*., 2007; Hollingsworth, 2007; Newmaster *et al*., 2009).

Furthermore, it should be acknowledged that a dedicated reference database is the core step to reduce the influence of misidentification. In this study, we grouped misidentification cases into two categories for which the implications could lead to different consequences in a vegetational analysis. At first glance, cases of NI could be interpreted as the worst case, but they could be resolved by increasing the database population. By contrast, cases of BI represent the critical point in a DNA barcoding context because they can completely alter the results of vegetational analysis.

At the present state of the art, DNA barcoding efficacy is quite different when applied to animals and plants. In general, this approach works well among metazoans (Hebert *et al*., 2003), whereas, in plants, it suffers from the problems discussed above (e.g. DNA amplification, BLAST identification, poorly populated databases) and recently highlighted at the Fourth International Barcode of Life Conference (http://www.dnabarcodes2011.org/). However, in several cases, even a DNA barcoding approach characterized by a reduced discriminatory capacity can be useful (Hollingsworth *et al*., 2011). One of these cases is the discrimination of plant species in a local flora (Burgess *et al*., 2011), in which species are usually phylogenetically distant and, consequently, easily identifiable. However, in a local flora, rare, endemic or alien species may be present without being represented in the molecular databases. As suggested by our study, a tentative solution is the development of a local dedicated database. In some way, this approach is similar to the identification workflow proposed by KeyToNature (http://www.keytonature.eu/), where complex morphological taxonomic keys are simplified in order to fit better with the local flora, therefore reducing cases of BI and NI. In our opinion, the combination of morphological traits with molecular data will substantially improve the plant identification systems.

In conclusion, we applied DNA barcoding in a practical context in which molecular investigations are

Table 3. Efficacy of a BLAST search approach in the identification of species samples collected in the context of this study for the three tested barcode regions

		trnH-psbA		*matK*		*rbcL*	
Code	Name	GenBank	BLAST-ID	GenBank	BLAST-ID	GenBank	BLAST-ID
MIB:Zpl:01671	*Inula hirta* L.	NO	BI	NO	NI	NO	NI
MIB:Zpl:01672	*Inula salicina* L.	OK	BI	NO	NI	NO	NI
MIB:Zpl:01683	*Geranium sanguineum* L.	NO	NI	NO	–	OK	BI
MIB:Zpl:01686	*Carex humilis* Leyser	NO	NI	NO	NI	NO	NI
MIB:Zpl:01692	*Dactylis glomerata* L.	OK	ID	OK	ID	OK	BI
MIB:Zpl:01702	*Teucrium montanum* L.	NO	NI	NO	NI	NO	NI
MIB:Zpl:01703	*Asperula purpurea* (L) Erhend	NO	NI	NO	–	NO	NI
MIB:Zpl:01705	*Centaurea bracteata* Scop.	NO	NI	NO	–	NO	BI
MIB:Zpl:01709	*Globularia cordifolia* L.	NO	BI	OK	ID	OK	ID
MIB:Zpl:01714	*Dorycnium pentaphyllum* Scop.	NO	NI	NO	–	NO	NI
MIB:Zpl:01718	*Teucrium chamaedrys* L.	NO	NI	NO	NI	OK	BI
MIB:Zpl:01721	*Origanum vulgare L.*	OK	ID	OK	ID	OK	BI
MIB:Zpl:02012	*Viola hirta* L.	NO	NI	NO	NI	NO	NI
MIB:Zpl:02013	*Pimpinella saxifraga* L.	OK	ID	OK	ID	OK	BI
MIB:Zpl:02014	*Bromus erectus* Huds.	NO	BI	OK	ID	OK	BI
MIB:Zpl:02016	*Hieracium murorum* L.	NO	NI	NO	–	NO	NI
MIB:Zpl:02017	*Sesleria varia* (Jacq.) Wettst.	NO	NI	OK	BI	OK	ID
MIB:Zpl:02018	*Arabis hirsuta* (L) Scop.	OK	ID	OK	ID	OK	BI
MIB:Zpl:02019	*Campanula rotundifolia* L.	NO	NI	OK	ID	OK	BI
MIB:Zpl:02020	*Viola reichenbachiana* Jord. ex Boreau	NO	NI	NO	–	NO	NI
MIB:Zpl:02021	*Brachypodium rupestre* (Host) Roem. & Schult.	NO	BI	NO	–	NO	NI
MIB:Zpl:02022	*Coronilla eremus* L.	NO	NI	NO	–	OK	BI
MIB:Zpl:02027	*Prunella grandiflora* Jacq.	NO	NI	NO	–	OK	BI
MIB:Zpl:02028	*Corylus avellana* L.	OK	BI	OK	BI	OK	BI
MIB:Zpl:02029	*Aster amellus* L.	NO	NI	NO	–	NO	BI
MIB:Zpl:02032	*Helianthemum nummularium* (L.)	NO	BI	NO	–	NO	BI
MIB:Zpl:02033	*Rubus fructicosus* L. agg.	NO	–	NO	–	NO	BI
MIB:Zpl:02034	*Allium sphaerocephalon* L.	NO	–	NO	–	NO	NI
MIB:Zpl:02035	*Briza media* L.	NO	–	OK	ID	OK	BI
MIB:Zpl:02799	*Anthericum liliago* L.	NO	NI	OK	ID	OK	BI
MIB:Zpl:02801	*Erica carnea* L.	NO	NI	OK	ID	NO	NI
MIB:Zpl:02802	*Fumana procumbens* (Dunal) Gren. & Godr.	NO	NI	NO	–	NO	NI
MIB:Zpl:02803	*Leontodon hispidus* L.	NO	NI	OK	BI	OK	ID
MIB:Zpl:02807	*Chrysopogon gryllus* (L.) Trin.	NO	BI	NO	–	NO	NI
MIB:Zpl:02808	*Carlina acaulis* L.	OK	ID	NO	–	NO	BI
MIB:Zpl:02811	*Pteridium aquilinum* (L.) Kuhn	OK	ID	OK	ID	OK	BI
MIB:Zpl:02812	*Clematis vitalba* L.	OK	ID	OK	ID	OK	ID
MIB:Zpl:02814	*Polygala chamaebuxus* L.	NO	NI	OK	ID	OK	ID
MIB:Zpl:02810	*Galium lucidum* All.	NO	NI	NO	–	OK	BI
MIB:Zpl:03832	*Aquilegia atrata* W.D.J.Koch.	NO	NI	NO	–	NO	BI
MIB:Zpl:03979	*Mercurialis perennis* L.	OK	ID	OK	ID	OK	ID
MIB:Zpl:03837	*Hippocrepis comosa* L.	NO	NI	NO	–	NO	NI
MIB:Zpl:03839	*Juniperus communis* L.	OK	BI	OK	BI	OK	BI
MIB:zpl:01701	*Colchicum autumnale* L.	OK	ID	OK	ID	NO	NI
MIB:Zpl:03316	*Prunus spinosa* L.	OK	ID	OK	ID	OK	ID
MIB:Zpl:03525	*Sanguisorba minor* Scop.	OK	ID	OK	ID	NO	NI
MIB:Zpl:03205	*Tamus communis* (L.)	OK	ID	NO	–	OK	ID
MIB:Zpl:03522	*Tanacetum corymbosum* (L.) Sch.Bip.	NO	NI	NO	–	NO	NI
MIB:Zpl:03840	*Calamagrostis arundinacea* (L.) Roth	NO	NI	OK	BI	NO	NI

For each species (identified directly in the field with classical approaches), the availability of GenBank entries (GenBank) previously deposited and an index of identification success (BLAST-ID) are reported for all three markers. Identification categories are based on the maximum similarity scores provided in Table S2 (see Supporting Information) and are named as ID (identified), BI (bad identification) and NI (not identifiable) as stated in Material and Methods. –, no sequence obtained for the sample.

Table 4. Evaluation of costs and benefits of classical vegetational and DNA barcoding analyses

	Vegetational analysis	DNA barcoding analysis
Field work		
Floristic knowledge	Required	Not required
Plant sampling	Required	Required
Time consumed for field work	40 h (5 inspections)*	16 h (2 inspections)*
Laboratory work		
Floristic knowledge	Required	Not required
Knowledge of molecular biology techniques	Not required	Basic knowledge
DNA extraction	Not required	$0.50
DNA amplification and sequencing	Not required	$2.35 for each sample and for single marker
Time	16 h†	10 h‡
Type of collected data		
Qualitative data (species presence/absence)	Yes	Yes
Quantitative data (cover, number of individuals, etc.)	Yes	No
Influencing variables		
Number of evaluable plots	Strictly related to operator abundance	Less influenced by personnel availability
Identification success depending on botanical characteristics	Literature review or description of local flora. Absence of critical taxa or exotic species	Absence of hybrid taxa and closely related molecular taxa

*Each inspection was estimated in 8 h.
†Each morphological identification deserving herbarium comparisons and the use of dichotomous keys was estimated to be accomplished in 20 min (for a total of 49 species; see Material and Methods).
‡In the molecular laboratory, the 49 samples were processed simultaneously (DNA extraction, PCR amplification, DNA sequencing and BLAST search). The cost of molecular analysis was in agreement with the data described by Newmaster *et al.* (2009) and Hajibabaei *et al*. (2005).

uncommon. In our case, vegetational analysis on Festuco-Brometalia grasslands (Habitat 6210) showed that, to restore and maintain grassland plant biodiversity, shrub coverage must be removed (Barbaro, Dutoit & Cozic, 2001; Bisteau & Mahy, 2005; Klimkowska *et al*., 2010). However, it is possible that, following this kind of restoration, competitive herbaceous species (e.g. *C. humilis* and *S. varia*) may become dominant, and give rise to almost monospecific grasslands characterized by low biodiversity. As a result of the unpredictability of plant dynamics, DNA barcoding studies can be a useful tool to better evaluate the evolution of vegetation cover and the biodiversity follow-up of restoration activities (Barbaro *et al*., 2001; Schrautzer *et al*., 2009).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Accession numbers corresponding to DNA sequences of the three analysed markers (*trnH-psbA*, *mat*K and *rbc*L) for each considered species. –, sequence not obtained.

Table S2. Results of the BLAST search analysis (maximum identity scores) performed on the three tested markers (*trnH-psbA*, *matK*, *rbcL*) for the species collected in the context of this study. The first three nearest matches with species name, GenBank accession and maximum identity scores (%) are reported for each queried sample.

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