Int J Legal Med (2010) 124:595–603
DOI 10.1007/s00414-010-0447-3

ORIGINAL ARTICLE

Identification of poisonous plants by DNA barcoding approach

Ilaria Bruni · Fabrizio De Mattia · Andrea Galimberti · Gabriele Galasso · Enrico Banfi · Maurizio Casiraghi · Massimo Labra

Received: 22 January 2010 / Accepted: 9 March 2010 / Published online: 31 March 2010

Abstract The plant exposures are one of the most frequent poisonings reported to poison control centres. The diagnosis of intoxicated patients is usually based on the morphological analysis of ingested plant portions; this procedure requires experience in systematic botany, because the plant identification is based on few evident traits. The objective of this research is to test DNA barcoding approach as a new universal tool to identify toxic plants univocally and rapidly. Five DNA barcode regions were evaluated: three cpDNA sequences (trnH-psbA, rpoB and matK) and two nuclear regions (At103 and sqd1). The performance of these markers was evaluated in three plant groups: (1) a large collection of angiosperms containing different toxic substances, (2) congeneric species showing different degrees of toxicity and (3) congeneric edible and poisonous plants. Based on assessments of PCR, sequence quality and resolution power in species discrimination, we recommend the combination of plastidial and nuclear markers to identify toxic plants. Concerning plastidial markers, matK and trnH-psbA showed consistent genetic variability. However, in agreement with CBOL Plant Working Group, we selected matK as the best marker, because trnH-psbA showed some problems in sequences sizes and alignments. As a final and relevant observation, we also propose the combination of matK with a nuclear marker such as At103 to distinguish toxic hybrids form parental species. In conclusion, our data support the claim that DNA barcoding is a powerful tool for poisonous plant identifications.

Keywords DNA barcoding · Poisonous plants · matK · trnH-psbA · rpoB · At103 · sqd1

Electronic supplementary material The online version of this article (doi:10.1007/s00414-010-0447-3) contains supplementary material, which is available to authorized users.

I. Bruni · F. De Mattia · A. Galimberti · M. Casiraghi · M. Labra (✉)
ZooPlantLab, Dipartimento di Biotecnologie e Bioscienze,
Università degli Studi di Milano-Bicocca,
Piazza della Scienza 2,
20126 Milan, Italy
e-mail: massimo.labra@unimib.it

G. Galasso · E. Banfi
Museo di Storia Naturale di Milano,
Corso Venezia 55,
20121 Milan, Italy

Introduction

The human diet includes a lot of domesticated plant species (for instance *Hordeum vulgare* L., *Zea mays* L., *Oryza sativa* L., etc.) derived from an evolutionary continuum of people–plants interactions, leading to plants showing higher yield, easier cultivation and detoxification from natural poisonous metabolites [37]. Since the beginning of the domestication, around 10,000 years ago, plants and derived were also used in medicine, and in the last years, the nutritional therapies and phytotherapy have emerged as new concepts and healing systems. However, several spontaneous plants are potentially toxic for human beings [27, 32, 34, 36]. The accidental ingestion of toxic plant portions (for instance seed, fruit, root, etc.) can cause severe poisoning or even death [24, 34]. In the last years, the plant exposures are among the most frequent poisoning cases reported by poison control centres [25]. Exposures to plants containing oxalate crystals, such as *Philodendron* and *Dieffenbachia*, are one of the most common toxic plant exposures reported in the USA [1]. The same toxic compounds were also present in several African species such as *Dieffenbachia*

amoena, *Alocasia macrorrhiza* and *Monstera deliciosa* [8] and in spontaneous or ornamental plant from other countries. To further complicate the picture, the amount of poison in a plant tends to vary with location, age of the plant, season and weather conditions [10, 18, 35].

The clinical diagnosis of intoxicated patients is typically based on the morphological analysis of plant fragments in the stomach contents [20]. This method is very tedious to perform, requires a considerable amount of training and usually a variable proportion of plant fragments remains unidentifiable. In addition, the plant species identifications can be difficult without residuals showing distinctive taxonomic elements. Recently, a new technique for plant identification based on the analysis of a short, standardised DNA region known as "DNA barcoding" ([13]; www.barcoding.si.edu) has been proposed. The basic idea of the discrimination system is simple: the sequence variation of the DNA barcode must be high enough between species, so that they can be discriminated from one another. In practice, a DNA sequence from such a standardised DNA region can be generated from a small tissue sample taken from an unidentified organism and can be compared to a library of reference sequences from described species. A match of the sequence from the unknown organism to one of the reference sequences can provide a rapid and reproducible identification [2, 13].

However, the selection of a barcode locus is complicated by the trade-off that arises between the need for universal application and maximal rates of sequence divergence [17]. A portion of the mitochondrial coxI (cytochrome *c* oxidase subunit 1) gene was largely applied as DNA barcode sequence for animal identification [13], but in land plants, this sequence is highly invariant and therefore unsuitable for use as DNA barcodes. Several alternative regions have been proposed, such as the plastidial conserved rpoB, rpoC1, rbcL and matK genes, several intergenic plastidial spacers (trnH-psbA, atpF-atpH and psbK-psbI) and the nuclear internal transcribed spacers [3, 9, 17]. A lot of research groups have proposed different combinations of these loci to increase the efficiency in species identification, especially for the critical groups [30]. However, both the identity and the number of the most appropriate regions for plant DNA barcoding remain contentious [14, 15].

The objective of the current study is to evaluate the universal application of DNA barcoding approach to univocally identify toxic plants starting from different plant portions. In this study, we provide an evaluation of the performance of five candidate barcoding loci on a selected group of poisonous plant species. The final objective is to develop a rapid molecular tool for toxic plants identification starting from a small portion of plant tissue from different biological samples such as stomach contents and/or faeces of patients. This tool could be useful for poison centres to identify the ingested plants univocally and to define the suitable medical treatments rapidly.

Materials and methods

Taxonomic sampling

A total of 50 land-plant species showing different poisonous substances was selected to test the performance of different candidate genomic regions for DNA barcoding analyses empirically. A complete list of investigated species is given in Table 1. Our sampling strategy was designed to have sufficient density of sampling within angiosperm, such that sets of closely related species are included as well as plants of divergent genera or families. Our taxon set was divided in three groups.

Group I: Hornamental plants as well as spontaneous plants containing different toxic substances (Table 1) Several of these show attractive poisonous portions such as fruits or flowers, and these are accidentally eaten, mainly by children. If the amount of ingested poisonous substance reaches significant levels in the body, clear symptoms of poisoning were highlighted. These samples were selected, because they represent a diverse set of species across the angiosperms (including monocots and eudicots) with various levels of phylogenetic distance. This provides a sound assessment of universality of the tested DNA barcoding markers.

Considering that the goal of our work is to set up a practical DNA barcoding approach suitable to identify poisonous plants starting from a small portion accidentally eaten, in this group, DNA was extracted from standard materials, such as leaves, and also from fruits-seeds that represented the accidentally eaten poisonous structures (Table 1).

Group II: Phylogenetically related plants showing different poisonous portions and degrees of toxicity were selected to evaluate the discriminating power of different DNA barcoding markers among closely related taxa In this group, species of the genera *Aconitum* (IIa) and *Sambucus* (IIb) were included.

Group III: Congeneric edible and poisonous plant species In the clinical diagnosis of intoxicated patients, it is very important to distinguish fragments of edible plants from the poisonous species elements. For this reason, some species of *Solanum* (group IIIa) and *Prunus* (group IIIb), two large angiosperm genera constituted by edible and poisonous plants, were analysed by different DNA barcoding markers. The final goal was to test the power of DNA barcoding to

Table 1 List of analysed plants divided in different groups (Gr) (see "Materials and methods")

No.	Gr	V. N.	Species	Family	Common name	Poisonous organ	Toxic substances	DNA from
1	I	MIB:zpl:01657	*Nandina domestica* Thunb.	Berberidaceae	Sacred Bamboo	Fruit and other part	Hydrocyanic acid and nandenine	L–F
2	I	MIB:zpl:01658	*Ilex aquifolium* L.	Aquifoliaceae	Holly	Fruit, leaves and seed	Theobromine, alkaloid and glucoside	L–F
3	I	MIB:zpl:01659	*Aucuba japonica* Thunb.	Garryaceae	Spotted-laurel	Fruit, leaves	Aucubin and different glycosides	L–F
4	I	MIB:zpl:01660	*Arum italicum* Mill.	Araceae	Lords-and-Ladies	All parts	Calcium oxalate crystals	L
5	I	MIB:zpl:01661	*Arum maculatum* L.	Araceae	Lords-and-Ladies	All parts	Calcium oxalate crystals	L
6	I	MIB:zpl:01662	*Convallaria majalis* L.	Ruscaceae	Lily-of-the-valley	All parts	Cardiac glycosides and saponins	L
7	I	MIB:zpl:01664	*Ruscus aculeatus* L.	Ruscaceae	Butcher's-broom	Fruit	Unknown	L
8	I	MIB:zpl:01665	*Hedera helix* L.	Araliaceae	Common Ivy	All parts	Triterpenoid saponins and polyacetylene	L
9	I	MIB:zpl:01666	*Hedera hibernica* (G.Kirchn.) Bean.	Araliaceae	Irish Ivy	All parts	Triterpenoid saponins and polyacetylene	L
10	I	MIB:zpl:01668	*Ligustrum vulgare* L.	Oleaceae	European Privet	Berries	Ligustrin, syringin and other glycosides	L
11	I	MIB:zpl:01669	*Ligustrum lucidum* W.T.Aiton	Oleaceae	Glossy Privet	Berries	Ligustrin, syringin and other glycosides	L
12	I	MIB:zpl:01673	*Ligustrum japonicum* Thunb.	Oleaceae	Japanese Privet	Berries	Ligustrin, syringin and other glycosides	L
13	I	MIB:zpl:01674	*Phytolacca americana* L.	Phytolaccaceae	American Pokeweed	All parts	Phytolaccatoxin and related triterpene saponins, alkaloid and histamines	L-F
14	I	MIB:zpl:01695	*Ficus benjamina* L.	Moraceae	Weeping Fig	Plant sap from all parts	Furocoumarins, psoralens, ficin	L
15	I	MIB:zpl:01697	*Monstera deliciosa* Liebm.	Araceae	Mexican Breadfruit	All parts	Needle-like calcium oxalate crystals and other unidentified toxins	L
16	I	MIB:zpl:01698	*Philodendron* sp.	Araceae	Philodendron	All parts	Calcium oxalate crystals and other toxins	L
17	I	MIB:zpl:01669	*Dieffenbachia seguine* (Jacq.) Schott	Araceae	Dumb Cane	All parts	Calcium oxalate crystals, oxalic acid	L
18	I	MIB:zpl:01706	*Spathiphyllum wallisii* Regel	Araceae	Peace-lily	Leaves	Calcium oxalate crystals	L
19	I	MIB:zpl:01707	*Trachelospermum jasminoides* Lem.	Apocynaceae	Star Jasmine	Leaves	Unknown	L
20	I	MIB:zpl:01708	*Schefflera arboricola* (Hayata) Merr.	Araliaceae	Schefflera	Leaves, plant sap	Oxalates	L
21	I	MIB:zpl:01710	*Sansevieria trifasciata* Prain	Ruscaceae	Snake Plant	All parts	Possibly saponins and organic acids	L
22	I	MIB:zpl:01711	*Hydrangea macrophylla* (Thunb.) Ser.	Hydrangeaceae	Hydrangea	All parts	Cyanogenic glycoside such as hydrangin	L
23	I	MIB:zpl:01712	*Wisteria sinensis* (Sims) Sweet	Fabaceae	Chinese Wisteria	Seed, and other parts	Glycoside (i.e. wisterin) and a toxic resin	L
24	I	MIB:zpl:01713	*Nerium oleander* L.	Apocynaceae	Oleander	All parts, green or dry	Glycosides, nerioside, oleandroside, ecc	L
25	I	MIB:zpl:01715	*Skimmia reevesiana* (Fortune) Fortune	Rutaceae	Skimmia	Fruit	Alkaloid called "skimmianin"	L
26	I	MIB:zpl:01716	*Kalanchoë daigremontiana* Hamet & Perrier	Crassulaceae	Mexican Hat Plant	Leaves, stems	Glycoside such as daigremontianin	L
27	I	MIB:zpl:01717	*Anthurium andraeanum* Linden	Araceae	Tail Flower	All parts	Calcium oxalate crystals	L
28	I	MIB:zpl:01719	*Veratrum lobelianum* Bernh.	Melanthiaceae	False-helleborine	All parts	Steroidal alkaloids	L
29	I	MIB:zpl:01720	*Veratrum nigrum* L.	Melanthiaceae	Black False-helleborine	All parts	Steroidal alkaloids	L
30	I	MIB:zpl:01722	*Lycianthes rantonnetii* (Carrière) Bitter	Solanaceae	Blue Potato-bush	All parts	Different alkaloids	L
31	I	MIB:zpl:01663	*Atropa bella-donna* L.	Solanaceae	Deadly Nightshade	All parts, mainly berries	Tropane alkaloids, atropine and others	L
32	I	MIB:zpl:01701	*Colchicum autumnale* L.	Colchicaceae	Meadow Saffron	All parts	Alkaloid colchicine	L
33	I	MIB:zpl:01676	*Aconitum lycoctonum* L.	Ranunculaceae	Wolf's-bane	All parts	Alkaloids aconitine and others	L

Table 1 (continued)

No.	Gr	V. N.	Species	Family	Common name	Poisonous organ	Toxic substances	DNA from
34	I–IIa	MIB:zpl:01675	*Aconitum napellus* L.	Ranunculaceae	Monk's-hood	All parts	Alkaloids aconitine and others	L
35	IIa	MIB:zpl:01670	*Aconitum degenii* Gáyer subsp. *paniculatum* (Arcang.) Mucher	Ranunculaceae	Panicled Monk's-hood	All parts	Alkaloids aconitine and others	L
36	IIa	MIB:zpl:01700	*Aconitum anthora* L.	Ranunculaceae	Pyrenean Monk's-hood	All parts	Alkaloids aconitine and others	L
37	I–IIb	MIB:zpl:01678	*Sambucus ebulus* L.	Adoxaceae	Dwarf Elder	Fruit; and other parts	Cyanogenic glycoside and others	L + F
38	IIb	MIB:zpl:01679	*Sambucus racemosa* L.	Adoxaceae	Red-berried Elder	Edible fruit	Cyanogenic glycoside in vegetative parts	L
39	IIb	MIB:zpl:01680	*Sambucus nigra* L.	Adoxaceae	Elder	Edible fruit	Cyanogenic glycoside in vegetative parts	L + F
40	IIIa	MIB:zpl:01689	*Solanum dulcamara* L.	Solanaceae	Bittersweet	Berries	Solanine and other alkaloids	L + F
41	IIIa	MIB:zpl:01690	*Solanum nigrum* L.	Solanaceae	Black Nightshade	Berries	Solanine and other alkaloids	L
42	I–IIIa	MIB:zpl:01691	*Solanum pseudocapsicum* L.	Solanaceae	Jerusalem-cherry	Fruits, other parts	Alcaloid such solanocapsine	L + F
43	IIIa	MIB:zpl:01693	*Solanum lycopersicum* L.	Solanaceae	Tomato	Edible fruit	Not toxic	L
44	IIIa	MIB:zpl:01694	*Solanum tuberosum* L.	Solanaceae	Potato	Edible fruit	Not toxic	L
45	IIIb	MIB:zpl:01681	*Prunus armeniaca* L.	Rosaceae	Apricot	Edible fruit	Seeds contain cyanogenic glycoside	L
46	IIIb	MIB:zpl:01682	*Prunus avium* L.	Rosaceae	Cherry	Edible fruit	Seeds contain cyanogenic glycoside	L
47	IIIb	MIB:zpl:01684	*Prunus cerasus* L.	Rosaceae	Sour Cherry	Edible fruit	Seeds contain cyanogenic glycoside	L
48	IIIb	MIB:zpl:01685	*Prunus domestica* L.	Rosaceae	Plum	Edible fruit	Seeds contain cyanogenic glycoside	L
49	I–IIIb	MIB:zpl:01687	*Prunus laurocerasus* L.	Rosaceae	Cherry Laurel	Vegetative parts, fruit and seed	Cyanogenic glycoside, amygdalin and other	L
50	IIIb	MIB:zpl:01688	*Prunus persica* (L.) Batsch	Rosaceae	Peach	Edible fruit	Seeds contain cyanogenic glycoside	L

For each species, the voucher number (V.N.) and the taxonomic characteristic were given and was provided details of the toxicity (poisonous organ and toxic substances)

L leaves, *F* fruits

distinguish common edible fruits from fragments of toxic plants accidentally ingested.

DNA isolation and amplification

Young leaves or fruits-seeds (100 mg) of the 50 selected plants were used for DNA analysis. DNA was isolated using the DNeasy Isolation and Purification kit (Qiagen, Milan, Italy) to obtain high-quality DNA, free of polysaccharides or other metabolites that might interfere with DNA amplification. Purified DNA concentration of each sample was estimated both fluorometrically and by comparison of ethidium bromide-stained band intensities with λ DNA standard.

To assess the comparative performance of different DNA markers, each DNA sample (Table 1) was analysed with all the five candidate DNA barcoding regions. These included two coding (rpoB and matK) and one non-coding (trnH-psbA intergenic spacer) plastidial DNA region, that feature in the preferred barcode solutions of different research groups [14, 15]. Two additional nuclear DNA regions were also analysed to evaluate the performance of nuclear genome in comparison with plastidial one. These regions are the sqd1 (UDP sulfoquinovose synthase) and At103 (Mg-protoporphyrin IX monomethyl ester cyclase) developed by Li et al. [22] as universally amplifiable markers for phylogenetic reconstructions.

PCR for each candidate marker 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 for 7 min at 94°C, 35 cycles of denaturation (45 s at 94°C), annealing (30 s at different temperature; see Table 2) and extension (1 min at 72°C), and a final extension at 72°C for 7 min. Details of primers used for amplification are given in Table 2. Products were submitted for sequence analysis to Macrogen (http://www.macrogen.com). The heavy DNA strands were bidirectionally sequenced using an ABI 155 3730XL automated sequencer at Macrogen Inc., Korea. Manual editing of raw traces and subsequent alignments of forward and reverse sequences enabled us to assign edited sequences for most species. The 3′ and 5′ terminals were clipped to generate consensus sequences for each taxon.

Data analysis

Our criterion for assessing universality of the five DNA candidate markers involves the establishment of which regions could be routinely amplified and sequenced in the maximum number of analysed plants. We have tested only the most universal primer combinations (Table 2) for each candidate DNA marker to facilitate the interpretation of successes and failures. 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 template. The second stage included only samples that did not amplify or that produced multiple PCR products. Samples of both types of failure were re-amplified using 1 and 25 ng of DNA template. The samples that failed to amplify were repeated at lower stringency (with a reduction of 5°C in the annealing temperature as described in Table 2) and 40 PCR cycles. Only in case of negative amplification in all conditions the PCR reaction was considered a failure.

Each marker was also evaluated, including the sequence length and the alignment success in all the analysed plant groups. Evaluation of comparative levels of variation and discrimination of different five markers were undertaken using MEGA 4.0 [31] to generate Kimura 2-parameter (K2P) distance matrices for each locus. K2P distances were used following guidelines from the Consortium for the Barcoding of Life to evaluate performance among barcoding loci (http://www.barcoding.si.edu/protocols.html). The discrimination values (K2P sequences divergence converted into percent) were computed for each locus in all the analysed groups.

Table 2 Details of primers used for amplification of the five candidate DNA barcoding markers

Locus	Primer name	Sequences (5′–3′)	Annealing temperature (°C)	Ref.
Nuclear Sqd1	Sqd1 F Sqd1 R	CTTGGGACSATGGGTGARTATGG CCWACAGCAGCYTGMACACAGAACC	63	[22]
Nuclear At103	At103 F At103 R	CTTCAAGCCMAAGTTCATCTTCTA TTGGCAATCATTGAGGTACATNGTMACATA	55	[22]
Plastidial matK	matK 390 matK 1326	CGATCTATTCATTCAATATTC TCTAGCACACGAAAGTCGAAGT	50	[5]
Plastidial trnH-psbA	psbA trnH	GTTATGCATGAACGTAATGCTC CGCGCATGGTGGATTCACAATCC	53	[26]
Plastidial rpoB	rpoB 1F rpoB 4R	AAGTGCATTGTTGGAACTGG GATCCCAGCATCACAATTCC	55	[9]

Results

Amplification and sequencing success

A total of 50 poisonous plants (Table 1) was used for DNA extraction. High DNA quality was obtained from all the analysed samples. For nine taxa (Table 1), the DNA was extracted both from leaves and fruits-seeds; however, no difference was observed in the PCR success, sequencing quality and sequences alignments for the template DNA obtained from different plant portions.

Results of our DNA barcoding analysis across the three selected plant groups showed a conspicuous difference among the five tested loci with respect to amplification success, PCR products lengths and sequences quality (Table 3). All the three plastidial markers exhibited high PCR success with standard primers with 10 ng of DNA as template for DNA amplification; non-relevant differences were detected among the three plant groups (Table 3). In the case of nuclear markers, only the At103 was successfully amplified in the standard PCR condition (10 ng of DNA as template) for a large number of plants of our dataset, while sqd1 showed some amplification problems for several samples of group I and IIb (Table 3) both starting from 1, 10 and 25 ng of DNA.

All the PCR products corresponding to the five DNA markers were easily sequenced, with exclusion of four samples (Table 3). The evaluation of the sequence quality from the tested loci demonstrated that high-quality bidirectional sequences were routinely obtained from almost all loci. Success rates for bidirectional sequences were highest for plastidial markers. The greatest problems in obtaining bidirectional sequences with few ambiguous bases were encountered with the At103 sequences in part attributable to a high frequency of mononucleotide repeats disrupting individual sequencing reads. This required more manual

Table 3 Summary of the proportion of individuals successfully amplified and sequenced and divergence values from five candidate barcoding regions in three analysed groups of poisonous plants

	Group	trnH-psbA	Mat K	RpoB	Sqd1	At103
Amplification success	I	34/37	36/37	36/37	17/37	29/37
	IIa	4/4	4/4	4/4	2/4	2/4
	IIb	3/3	3/3	3/3	1/3	3/3
	IIIa	5/5	5/5	5/5	5/5	5/5
	IIIb	6/6	6/6	6/6	6/6	5/6
Sequencing success	I	33/37	35/37	33/37	17/37	26/37
	IIa	4/4	4/4	4/4	2/4	2/4
	IIb	3/3	3/3	3/3	1/3	3/3
	IIIa	5/5	5/5	5/5	5/5	5/5
	IIIb	6/6	6/6	6/6	6/6	5/6
Sequence length	I	210–472	788–850	416–479	237–262	299–730
	IIa	218–238	781–791	477	237	302–364
	IIb	423–454	771–788	417	–	305–478
	IIIa	470–486	837–846	471	262	326–351
	IIIb	358–364	850	474	255	482
Alignment success	I	NA	35/37	33/37	16/37	26/37
	IIa	4/4	4/4	3/4	2/4	2/4
	IIb	3/3	3/3	3/3	1/3	3/3
	IIIa	5/5	5/5	5/5	5/5	5/5
	IIIb	6/6	6/6	6/6	6/6	5/6
Alignment length	I	NC	825	423	237	234
	IIa	240	787	477	237	300
	IIb	454	767	417	–	166
	IIIa	501	846	471	262	230
	IIIb	380	850	474	255	482
% of divergence	I	NC	22.8	11.8	19.7	17,8
	IIa	6.3	1.4	0.2	1.3	2.2
	IIb	7.6	0.2	0.2	–	17.5
	IIIa	6.3	1.4	1.5	3.3	9.9
	IIIb	6.7	1.1	4.9	7.5	2.3

In the samples of the group IIb, the sqd1 sequences have been not successful amplified

NC not computable, *NA* not alignable

editing. In order to properly assess the performance of this marker, only the first 250 bp of sequence was used for statistical analyses.

The accession numbers of the considered sequences are listed in Table 5 of the Electronic supplementary materials.

Evaluation of the discrimination value of the five candidate loci

The sequence divergence values for each tested locus were computed to define which is the best DNA barcode marker for poisonous plants identification (Tables 3 and 4). The trnH-psbA ranked first in divergence value in the analysed groups compared with the other plastid and nuclear regions. However, the high variability of this DNA spacer did not allow to align properly the sequences related to species of the group I and to define the genetic diversity values (Table 3).

The matK gene showed consistent genetic variability among samples of group I and IIa; however, the divergence among the plants of group IIb was very low: *Sambucus ebulus* L. and *Sambucus nigra* L. showed the same matK sequence (Table 4). This marker is also able to distinguish edible from poisonous plants of group III (Table 4).

The rpoB sequences showed the lower sequence divergences than the other four tested markers. The efficacy of this marker is very limited in several groups such as IIa and IIb (Tables 3 and 4); however, edible plants of group IIIb were clearly distinguished from toxic species (i.e. *Prunus laurocerasus* L.).

Nuclear markers showed consistent genetic variability among all analysed groups; however, we underline that sqd1 was amplified in a limited number of species, and in group IIa, sequences of this gene showed a large conserved trait. On the contrary the At103 showed a satisfactory level of amplification and a wide genetic variability in all the analysed groups. This marker seems also to allow a good distinction among congeneric species, including toxic and edible species included in groups IIIa and IIIb.

The results from data-mining sequences in GenBank by using BLAST analysis notwithstanding their relatively crude nature search engine indicated that matK and trnH-psbA were successful at returning a correct match for 24 and 22 samples, respectively (data not shown). This suggests that generic and public databases such as NCBI or EMBL have already catalogued numerous matK and trnH-psbA sequences referable to poisonous plants species. The other three loci had a few too many sequences in GenBank to conduct a robust test (both rpoB and sqd1 showed three correct matches, while no-corresponding sequence for the At103 was found in GenBank).

Table 4 Vale of sequence divergences (Kimura 2-parameter *d* value) computed between individuals of each of the congeneric group (IIa, IIb, IIIa and IIIb)

		trnH-psbA	Mat K	rpoB	Sqd1	At 103
Samples of group IIa						
33	34	0.061	0.018	0.004	0.026	0.013
33	35	0.093	0.019	0.004	0.004	0.024
33	36	0.024	0.013	0.002	0.004	0.034
34	35	0.048	0.001	0	0.021	0.020
34	36	0.051	0.016	0.002	0.021	0.031
35	36	0.103	0.017	0.002	0	0.010
Samples of group IIb						
37	38	0.098	0.003	0	–	0.136
37	39	0.091	0	0.002	–	0.181
38	39	0.038	0.003	0.002	–	0.206
Samples of group IIIa						
40	41	0.046	0.012	0	0.012	0.194
40	42	0.090	0.018	0.015	0.027	0.194
40	43	0.053	0.016	0.024	0.052	0.201
40	44	0.039	0.011	0.019	0.031	0.183
41	42	0.091	0.019	0.015	0.023	0.031
41	43	0.057	0.013	0.024	0.044	0.036
41	44	0.044	0.008	0.019	0.027	0.031
42	43	0.103	0.020	0.015	0.052	0.050
42	44	0.086	0.016	0.013	0.035	0.045
43	44	0.026	0.005	0.009	0.027	0.022
Samples of group IIIb						
45	46	0.006	0.016	0.006	0.016	0.023
45	47	0.009	0.006	0.008	0.016	0.019
45	48	0.020	0.007	0.004	0.008	0.015
45	49	0.018	0.013	0.131	0.185	0.043
45	50	0.174	0.004	0.004	0.024	0.013
46	47	0.006	0.011	0.002	0	0.019
46	48	0.026	0.013	0.011	0.008	0.017
46	49	0.021	0.022	0.128	0.206	0.043
46	50	0.157	0.014	0.011	0.016	0.015
47	48	0.026	0.005	0.008	0.008	0.019
47	49	0.023	0.013	0.131	0.206	0.023
47	50	0.159	0.006	0.008	0.016	0.015
48	49	0.033	0.013	0.136	0.196	0.043
48	50	0.157	0.006	0.004	0.016	0.006
49	50	0.178	0.012	0.136	0.210	0.039

The analysis was conducted for each of the five selected markers. The Sqd1 sequences were not obtained for the individuals of group IIb

Discussion

The main purpose of this work is to provide a rapid and accurate identification system of unidentifiable poisonous plants based on DNA analysis. DNA barcoding is promising in providing a practical, standardised, species-level

identification tool that can be used for different study including forensic analyses [7] and animal diet determination when the food is not identifiable by morphological criteria [7, 33]. Similar situations are found in the poisonous centres, where toxic plants should be identified starting from different portions of plant material (fruits, seed, leaves, etc.). In most cases, portions of the most attractive fruits and seeds, accidentally ingested from children, represent the only mean for species identification [20]. For these reasons, our research was conducted starting from different plant materials, including attractive fruits and seeds. DNA was extracted from plant samples collected in field or house and stored at room temperature without specific methods to preserve the DNA quality. DNA amplification and sequence analysis suggest that plant materials used for DNA extraction do not affect the results of DNA barcoding analysis; this supports the application of the DNA barcoding approach as a practical identification tool in the poisonous centres.

In our work, we tested the effectiveness of five different DNA barcode regions. The three plastidial markers provided a good performance; whereas, concerning the nuclear regions, At103 only showed a wide applicability in the analysed groups. Since an ideal DNA barcode should be applied to a large number of species with standard PCR conditions [4, 13], according to our results, the sqd1 gene was excluded among the candidate regions.

Among the three plastidial markers, all worked across the analysed groups with a single set of PCR conditions. This is an impressive performance given by the range of taxonomic diversity encompassed. However, the trade-off in the universality of the rpoB is its relatively low level of species discrimination power. For this reason, we consider this marker unsuitable for the poisonous plants identification. On the contrary, the non-coding trnH-psbA intergenic spacer is the most viable candidate in the analysed poisonous plants. PCR priming sites within highly conserved flanking sequences, combined with a non-coding region that exhibits high numbers of substitutions, made the trnH-psbA spacer highly suitable as a plant barcode. Previous researches suggested that this intergenic spacer has alignment problems, especially in some monocots [4] and conifers [14]. Our results confirm these problems (Table 3), even if the preliminary investigations carried out in GenBank showed good results for this marker, and overall, we concur with Kress and Erickson [16] that trnH-psbA is one of the most suitable plant DNA barcode.

The matK gene showed easy amplification and alignment in the analysed species and high level of discrimination values in several analysed groups (Tables 3 and 4). Lahaye et al. [19] identified this gene as a universal DNA barcode for flower plants. Recently, the CBOL Plant Working Group (http://www.barcoding.si.edu/plant_working_group.html) confirmed this prompt and suggested the combination of matK with rbcL as a universal plant DNA barcode. Although we did not test the rbcL because several publications underline the low discriminating power of this gene [9, 14, 26], our data are in agreement with the CBOL statement on the role of matK gene as a universal DNA barcode.

Concerning the nuclear markers, our analysis focused on the Conserved Ortologous Sequences genes [22]. Among these, At103 was amplified and sequenced in a large part of the tested samples and showed a consistent genetic diversity, supporting its application as a DNA barcode. We underline that nuclear markers can provide a more reliable assessment of hybridization than uniparentally inherited plastid DNA regions [3, 11], and this is very important in the plant kingdom where hybridization events are largely diffused. For example, *Sambucus* and *Prunus* are two important genera considered in our work showing different natural and commercial hybrids [23, 29]. Breeding events between poisonous and non-poisonous plants could result in individuals showing different degrees of toxic substances [12, 21, 28]. Only by the combination of the plastidial marker with the nuclear one is it possible to distinguish hybrids from parental species.

In conclusion, our data confirm the utility of DNA barcoding for plant poisonous species identification. Our results support the application of matK as the best plastidial marker. Among the nuclear sequences, we suggest At103 as the most suitable candidate; however, future analysis will be required to evaluate the performance of this marker in a large plant dataset.

The next step of this research is toward the establishment of a dedicated poisonous plants database where all species are described either by morphological and molecular (DNA barcoding) approaches. Our preliminary in silico tests suggested that the availability of deposited reference sequences of matK and trnH-psbA relative to toxic plants could enable a fast identification of poisonous species with high efficiency. The dedicated database supported by proper bioinformatics technologies [2] will lead to the development of an innovative tool suitable for a rapid identification of toxic plant by poisonous centres.

Acknowledgements We are grateful to Dr.ssa Franca Davanzo, Dr.ssa Mami Azuma, and Marco Festa for technical information and assistance in the field. This work was supported by Fondazione Cariplo with the project entitle “Dai geni all'ecosistema: il DNA barcoding come supporto innovativo per la protezione della biodiversità e l'analisi della funzionalità delle reti ecologiche” and by Regione Sardegna with the project entitle “Tassonomia integrata (morfologia e DNA barcoding) per la salvaguardia della biodiversità vegetale della Sardegna”.

References

1. Burrows GE, Tyrl RJ (2006) Handbook of toxic plants of North America. Blackwell, Ames, pp 55–307
2. Casiraghi M, Labra M, Ferri E, Galimberti A, De Mattia F (2010) DNA barcoding: a six-question tour to improve users' awareness about the method. Brief Bioinform. doi:10.1093/bib/bbq003
3. Chase MW, Salamin N, Wilkinson M et al (2005) Land plants and DNA barcodes: short-term and long-term goals. Philos Trans R Soc London [Biol] 360:1889–1895
4. Chase MW, Cowan RS, Hollingsworth PM, van den Berg C, Madriñán S, Petersen G et al (2007) A proposal for a standardised protocol to barcode all land plants. Taxon 56:295–299
5. Cuènoud P, Savolainen V, Chatrou LW, Powell M, Grayer RJ, Chase MW (2002) Molecular phylogenetics of Caryophyllales based on nuclear 18S rDNA and plastid rbcL, atpB and matK DNA sequences. Am J Bot 89:132–144
6. Dawnay N, Ogden R, McEwing R, Carvalho GR, Thorpe RS (2007) Validation of the barcoding gene COI for use in forensic genetic species identification. Forensic Sci Int 173:1–6
7. Deagle BE, Gales NJ, Evans K, Jarman SN, Robinson S, Trebilco R, Hindel MA (2007) Studying seabird diet through genetic analysis of faeces: a case study on *Macaroni penguins* (*Eudyptes chrysolophus*). PLoS ONE 2:e831
8. Edeoga HO, Ogbebor NO (1999) Distribution of calcium oxalate crystals in some Nigerian species of Aneilema R. Br. (Commelinaceae). Plant Biosys 133:193–198
9. Fazekas AJ, Burgess KS, Kesanakurti PR, Graham SW, Newmaster SG, Husband BC, Percy DM, Hajibabaei M, Barrett SC (2008) Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. PLoS ONE 3:e2802
10. Gardner DR, Pfister JA (2007) Toxic alkaloid concentrations in *Delphinium nuttallianum*, *Delphinium andersonii*, and *Delphinium geyeri* in the Intermountain Region. Rangeland Ecol Manage 60:441–446
11. Grassi F, Labra M, Minuto L, Casazza G, Sala F (2006) Natural hybridization in *Saxifraga callosa* Sm. Plant Biol 8:243–252
12. Hammouda FM, Rizk AM, El-Missiry MM, Ghaleb HA, Madkour MK, Pohland AE, Wood G (1988) Poisonous plants contaminating edible ones and toxic substances in plant foods iv. phytochemistry and toxicity of *Lolium temulentum*. Pharm Biol 26:240–245
13. Hebert PDN, Cywinska A, Ball SL, DeWaard JR (2003) Biological identification through DNA barcodes. Proc R Soc Lond [Biol] 270:313–321
14. Hollingsworth ML, Clark A, Forrest LL, Richardson JE, Pennington RT, Long D, Cowan R, Chase MW, Gaudeul M, Hollingsworth PM (2009a) Selecting barcoding loci for plants: evaluation of seven candidate loci with species level sampling in three divergent groups of land plants. Mol Ecol Resour 9:439–457
15. Hollingsworth PM, Forresta LL, Spougeb JL et al (2009b) A DNA barcode for land plants. Proc Natl Acad Sci USA 106:12794–12797
16. Kress WJ, Erickson DL (2007) A two-locus global DNA barcode for land plants: the coding rbcL gene complements the non-coding trnH-psbA spacer region. PLoS ONE 2:e508
17. Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH (2005) Use of DNA barcodes to identify flowering plants. Proc Natl Acad Sci USA 102:8369–8374
18. Labra M, Miele M, Ledda B, Grassi F, Mazzei M, Sala F (2004) Morphological characterization, essential oil composition and DNA genotyping of *Ocimum basilicum* L. cultivars. Plant Sci 167:725–731
19. Lahaye R, Van der Bank M, Bogarin D et al (2008) DNA barcoding the floras of biodiversity hotspots. Proc Natl Acad Sci USA 105:2923–2928
20. Lawrence RA (1997) Poisonous plants: when they are a threat to children. Pediatr Rev 18:162–168
21. Li X, Ralphs MH, Gardner DR, Wang RRC (2002) Genetic variation within and among 22 accessions of three tall larkspur species (*Delphinium* spp.) based on RAPD markers. Biochem Syst Ecol 30:91–102
22. Li M, Wunder J, Bissoli G, Scarponi E, Gazzani S, Barbaro E, Saedler H, Varotto C (2008) Development of COS genes as universally amplifiable markers for phylogenetic reconstructions of closely related plant species. Cladistics 24:727–745
23. Liu W, Chen XS, Liu GJ, Liang Q, He TM, Feng JR (2007) Interspecific hybridization of *Prunus persica* with *P. armeniaca* and *P. salicina* using embryo rescue. Plant Cell Tissue Organ Cult 88:289–299
24. McIntire MS, Guest JR, Porterfield JF (1990) Philodendron—an infant death. J Toxicol Clin Toxicol 28:177–183
25. Mrvos R, Krenzelok EP, Jacobsen TD (2001) Toxidromes associated with the most common plant ingestions. Vet Hum Toxicol 43:366–369
26. Newmaster SG, Ragupathy S (2009) Testing plant barcoding in a sister species complex of pantropical Acacia (Mimosoideae, Fabaceae). Mol Ecol Resour 9:172–180
27. Ngogang J, Nkongmeneck BA, Biyiti Bi Essam LF, Essame Oyono JL, Tsabang N, Zapfack L, Ngono Mballa R, Tamze V (2008) Evaluation of acute and sub acute toxicity of four medicinal plants extracts used in Cameroon. Toxicol Lett 180:S185–S186
28. Savarese S, Andolfi A, Cimmino A et al (2009) Glycoalkaloids as biomarkers for recognition of cultivated, wild, and somatic hybrids of potato. Chem Biodiv 6:437–446
29. Simonovik B, Ivancic A, Jakse J, Bohanec B (2007) Production and genetic evaluation of interspecific hybrids within the genus Sambucus. Plant Breed 126:628–633
30. Starr JR, Naczi RFC, Chouinard BN (2009) Plant DNA barcodes and species resolution in sedges (Carex, Cyperaceae). Mol Ecol Resour 9:151–163
31. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA 4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599
32. Tinggi U (2003) Essentiality and toxicity of selenium and its status in Australia: a review. Toxicol Lett 137:103–110
33. Valentini A, Miquel C, Nawaz MA, Bellemain E, Coissac E, Pompanon F, Gielly L, Cruaud C, Nascetti G, Wincker P, Swenson JE, Taberlet P (2009) New perspectives in diet analysis based on DNA barcoding and parallel pyrosequencing: the trnL approach. Mol Ecol Resour 9:51–60
34. Vetter J (2000) Plant cyanogenic glycosides. Toxicon 38:11–36
35. Walker R (2004) Criteria for risk assessment of botanical food supplements. Toxicol Lett 149:187–195
36. Xia Q, Yan J, Chou MW, Fu PP (2008) Formation of DHP-derived DNA adducts from metabolic activation of the prototype heliotridine-type pyrrolizidine alkaloid, heliotrine. Toxicol Lett 178:77–82
37. Zohary D, Hopf M (1993) Domestication of plants in the old world. Clarendon, Oxford, pp 143–150