Academic Journal of Plant Sciences 2 (2): 82-91, 2009
ISSN 1995-8986

On the Taxonomy of *Laurus* L. (Lauraceae), Evidence from Isozymes, RAPD and ISSR

Abdel-Moneim Ibraheim Aboel-Atta

Biological and Geological Sciences Department, Faculty of Education,
Ain Shams University, Roxy, Heliopolis, P.C.11757, Cairo, Egypt

Abstract: Isozymes, RAPD and ISSR criteria were used to locate the variations between the Macaronesian taxa of *Laurus* namely *Laurus azorica* and *L. novocanariensis* and the continental *L. nobilis*. The recorded intraspecific polymorphism in the isozyme profiles were ascribed to occurrence of genetic mutation. The recorded polymorphism percentages between the studied taxa based on the used molecular markers showed that the ISSR markers yielded a higher polymorphism than those of RAPD. The study showed a closer relationship between *L. azorica* and *L. novocanariensis* than those between any of them and *L. nobilis*, thus questioning their recent split into two different species. However, the recorded similarity between the continental *L. nobilis* and *L. novocanariensis* was higher than those between the continental *L. nobilis* and *L. azorica*.

Key words: Isozymes, *Laurus,* Lauraceae, RAPD, ISSR, PCR, Taxonomy

INTRODUCTION

Laurus L. is a genus of evergreen trees belonging to the Lauraceae and comprises two species, *L. nobilis* L. and *L. azorica* (Seub.) Franco [1]. *Laurus nobilis* (laurel), either wild or cultivated, is distributed in regions of warm climate and high rainfall [2-4]; common in more humid microclimates such as tubes, canyons and valley funds. It extends in southern and Western Europe, including all the Mediterranean area, the Atlantic coast of France and the Iberian Peninsula. Jalas and Suominen [5] considered the populations of center and east Mediterranean basin only as wild laurel and estimated that populations in the Iberian Peninsula and western France could be the result of different introduction events [6]. *Laurus azorica* (Seub.) Franco, syn. *Laurus canariensis* Webb and Berth. non Willd. or Macaronesian laurel is native to Canary Islands, Cape Verde, Madeira and the Azores; common in Tenerife's laurel forests; mostly found in rocky precipices and by streams. It was also discovered in some enclaves in France, Iberian Peninsula and has been recorded in the Atlas Mountains of Northern Africa [7-5].

The diagnostic key characters of both *L. azorica* sensu lato and the continental *L. nobilis* often overlap mainly because of their marked leaf heterophylly and similarity in their habit [9], cuticular features of leaves [10] and flowers [11]. On the other hand, the macro-morphological variations used to differentiate both species are scarce and represented only by the texture of young branches and width of the leaves. Young branches and leaves are smoothly pubescent in *L. azorica*, while they are predominently glabrous in *L. nobilis*. Leaves are lightly built with leveled venation and have a softer lemon scent in *L. azorica* than in *L. nobilis* [12, 13]. Barbero *et al.* [7] and Morales *et al.* [14] consider that both *L. nobilis* and *L. azorica* are derived from a common ancestor broadly distributed in Europe from the Miocene until the Pleistocene. This ancestor was a component of the lauroid leaf forests that occupied Southern Europe and Northern Africa about 20 million years ago. Climatic changes taking place during the Quaternary era resulted in a gradual decrease of these populations all over their distribution area [14, 15]. Rohwer [16] suggested that, *Laurus azorica* might better be considered as a mere island subspecies of *L. nobilis*.

Arroyo-Garcia *et al.* [6] recorded greater similarities between populations of *L. azorica* in the Canary Islands and Madeira and the native *L. nobilis* of northern Spain than to populations of *L. nobilis* native to France and Italy. They concluded that, *Laurus* of Madeira and the Canary Islands could be derived from Iberian *L. nobilis* because of two recent independent colonisations. On the other hand, Bramwell and Bramwell [17], Rivas-Martínez *et al.* [18] and Costa *et al.* [19] reported that, the

Corresponding Author: Abdel-Moneim Ibraheim Aboel-Atta, Biological and Geological Sciences Department, Faculty of Education, Ain Shams University, Roxy, Heliopolis, P.C.11757, Cairo, Egypt

populations of Canary Islands and Madera have mostly narrow and generally homogenous lanceolate leaves, whereas those of Azores have mostly suborbicular leaves. They placed the populations of Canary Islands and Madera in a new third species viz. *Laurus novocanariensis* Rivas Mart., Lousã, Fern. Prieto, E. Díaz, J.C. Costa and C. Aguiar. and retained the name *Laurus azorica* only for the Azores populations. However, the taxonomic status of *Laurus* is still questioned by ecological and genetic evidence [20].

Isozymes, random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) have been extensively utilized to discriminate the taxa at specific and varietals levels [21, 22]. Isozymes are widely used for genotype identification to evaluate genetic integrity [23]. In the Lauraceae, isozyme data were used in the delimitation of some problematic taxa, deducing the relationships between them, studying the genetic structure of natural populations of a taxon, or merely the identification of different varieties of the same taxon [24-27]. Either RAPD or ISSR provide good taxonomic markers to determine the rates and patterns of change occurring in DNA or proteins; and to reconstruct the evolutionary history of genes and organisms [28, 29]. In the present work isozymes, RAPD and ISSR are used in order to: locate the genetic variations between the two Macaronesian taxa of *Laurus* viz. *L. azorica* and *L. novocanariensis* in addition to *L. nobilis* and comparing them with their geographic distribution and with their previous classification based on morphological traits.

MATERIALS AND METHODS

Samples of the present study were supplied by Dr. M. H. A. Loutfy from the botanical gardens given in Table 1. The investigated taxa included the two taxa of *Laurus* from Macaronesia sensu Rivas-Martínez *et al.* [18] viz. *L. azorica* and *Laurus novocanariensis* in addition to *L. nobilis*. Macro-morphological criteria and taxa identification were performed from the live specimens and further confirmed by the aid of Tutin [31] and Short [32]. Nomenclature and synonymy were confirmed from the Missouri Botanical Gardens nomenclatural database - TROPICOS [32] and the International plant name index - IPNI [33]. Voucher specimens have been deposited at the Herbarium of Biological and Geological Sciences Department, Faculty of Education, Ain Shams University, Egypt.

The Isozymes used were: α-and β-esteras (Est.), and Acid phosphatase (Acph.), Isozymes were separated in 10 % Native-polyacrylamide gel electrophoresis [34]. For isozyme extraction, 0.5 g of fresh leaves was homogenized in 500 µl extraction buffer using a mortar and pestle; centrifuged at 10 000 rpm for five minutes; the supernatant was kept at -20°C until use. For electrophoresis, 50 µl of extract was mixed with 25µl of treatment buffer and 50 µl of this mixture was applied to the well. In gels staining, protocols of Scandalios [35] were used for α and β-Est.; Wendel and Weeden [36] for Acph. Gels were washed two or three times with tap water; fixed in ethanol: 20% glacial acetic acid (9:11 v/v) for 24 hours; and photographed.

Table 1: The studied taxa and, their sources

Taxon	English name	Source of seeds
1. *Laurus azorica* (Seub) Franco Anais. Inst. Sup. Agron, (Lisboa) 23:96 (1960) = *L. canariensis* Webb and Berth.non Willd.Phyt. Canar. iii 229 (1836-1850) = *Persea azorica* Seub. Ft. Azor. 29 (1844)	Canary Island Laurel	Jardim Botβnico do Faial, Azores-Portugal
2 *Laurus novocanariensis* Rivas Mart., Lousã, Fern. Prieto, E. Días, J.C. Costa and C. Aguiar nom. nov. in Itinera Geobotanica 15 (2) 703 (2002). = *L. canariensis* Webb and Berth.non Willd. Phyt. Canar. iii 229 (1836-1850). = *Persea azorica* Seub. Ft. Azor. 29 (1844)	Canary Islands laurel	Jardin Botanico Canario 'Viera Y Clavijo' Las Palmas de Gran Canaria Spain
3) Laurus nobilis L. Sp. PI. 369 (1753)	Sweet bay, Bay laurel, Roman laurel, laurel of history, Poets laurel Bay tree	Orto Botanico Università Degli Studi di Napoli Federico II Facoltà di Scienze. Italy

Table 2: The studied Isozyme systems and the produced bands.

	Taxa		
Isozyme system	*L. azorica* (1)	*L. novocanariensis* (2)	*L. nobilis* (3)
α- est	+	+	+
	+	+	+
	-	-	+
	-	-	+
	-	-	+
β- est	-	+	+
	-	+	-
	+	-	+
	-	-	+
	-	-	+
	-	-	+
Acph	+	+	+
	-	-	+
	+	+	+
	+	-	+

In the molecular analysis, 11 primers for each of the two PCR-based techniques; RAPD and ISSR were used. DNA extraction was performed using protocols of Dellaporta *et al.* [37]. For RAPD analysis, five 10-*mer* random DNA oligonucleotide primers (Operon Technologies, Inc, USA) of arbitrary sequences were independently used in PCR reactions as described by Williams *et al.* [38]. Codes and sequences of these primers were listed in Table 2. Amplifications were performed in 50 µl reaction volume containing: 0.2 mM dNTBs, 1.5 mM $MgCl_2$, 5.0 µl 10X buffer, 0.2 µl Primer, 3.0 µl template DNA (50 ng/ µl) and 0.3 µl Taq DNA polymerase (5U/ µl). Each of the reaction mixtures was overlaid with a drop of light mineral oil per sample. Amplifications were carried out in Perkin Elmer thermocycler. The optimal conditions for PCR amplification were as follows: an initial 4 minutes denaturation step at 94°C followed by 37 cycles of 1 minute at 94°C, 1 minute at 37°C and 2 miutes at 72°C, with a final extension step at 72°C for 8 minutes. A volume of 15 µl of the RAPD products were electrophoresed in 1.2 % agarose gel and run was performed at 100 V for about 60 minutes in Pharmacia submarine (20cm x 20cm). The bands were visualized on UV trans-illuminator and photographed by Polaroid camera.

ISSR analysis was carried out in a total reaction of 50µl containing 0.2 mM (dNTPs), 1.5mM $Mgcl_2$, 5.0 µl 10X buffer, 0.2 µl primer (50 pmoles), 3.0 µl template DNA (50ng/ µl), 0.3 µl Taq DNA polymerase (5U/ µl), in sterile water up to 50 µl. PCR amplification was programmed to fulfill 40 cycles after an initial denaturation cycle for 4 min. at 94°C. Each cycle consisted of a denaturation step at 94°C for 1 min., an annealing step at 47°C for 1 min. and an elongation step at 72°C for 2 min. The primer extension segment was done for 7 min. at 72°C in the final cycle. The molecular analysis was performed at the Desert Research Center (DRC), Matariya Cairo, Egypt. Sequences are deposited at the Gene Bank of Plant Breeding unit of DRC.

For scoring the isozymes, RAPD and ISSR data, clear and distinct bands were scored as (+) or (-) for presence and absence, respectively. Differences in bands intensity among profiles of the different samples were not considered. For creating a data matrix for numerical analysis, the recorded characters were analyzed by the NTSYS program package using the UPGMA clustering method [39]. The relationships between the studied taxa, expressed by average taxonomic distance (dissimilarity), were demonstrated as phenograms.

RESULTS AND DISCUSSION

Photos of the produced banding patterns by application of the isozyme technique on the studied taxa of *Laurus* are shown in Fig. 1. Their scored bands are given in Table 2, whereas number and types of bands and percentage of the total polymorphism are given in Table 3. The photos of the produced banding patterns by application of the ISSR and RAPD techniques on the studied taxa of *Laurus* are shown in Figs. 2 and 3 respectively. Their used primers are given in Table 4; their scored bands are given in Tables 5 and 6 respectively, whereas the number and types of bands and percentage of the total polymorphism are given in Table 7 and the produced UPGMA phenograms are shown in Figs. 4a-d.

Table 3: Percentage of the total polymorphism of isozyme data.

Isozyme system	Monomorphic bands	Polymorphic bands	Total of bands	Polymorphism (%)
α- est	2	3	5	60%
β- est	0	6	6	100%
Acph	2	2	4	50%

Table 4: The used ISSR and RAPD primers.

ISSR		RAPD	
primer	sequence	primer	sequence
17898A	(CA)6 AG	A1	5-CAGGCCCTTC-3
17898B	(CA)6 GG	A4	5-AATCGGGCTG-3
844A	(CA)8 AC	A7	5-GAAACGGGTG-3
HB10	(GA)6 CC	B1	5-GTTTCGCTCC-3
HB11	(GT)6 CC	B7	5-CAGCACTGAC-3
HB13	(GAG)3GC	-	-

Table 5: RAPD scored bands.

RAPD primer	M. s	1	2	3
A1	2029	-	-	+
	1229	+	+	+
	1130	+	+	+
	990	-	+	+
	738	+	+	+
	626	+	+	+
	494	+	+	+
	356	-	-	+
	300	+	+	+
	288	-	+	-
	182	+	+	+
	141	-	+	-
	84	-	+	+
	73	+	+	+
	68	-	+	+
A4	4000	+	+	+
	2031	+	+	+
	1834	+	+	+
	1338	+	+	+
	1114	+	+	+
	943	-	+	+
	871	+	-	-
	743	+	+	+
	657	+	+	+
	494	+	+	+
	413	+	-	-
	363	+	-	+
	319	+	+	+
	182	+	+	+
	116	+	+	+
	90	+	+	-
	87	-	+	+
	68	+	+	+

Table 5: Continued

A7	3676	-	+	-
	1943	-	-	+
	1600	+	-	-
	1429	-	+	+
	1171	-	+	+
	1057	-	+	+
	871	-	-	+
	729	+	+	+
	427	-	+	+
	409	+	-	-
	379	-	+	+
	292	+	+	+
	202	-	+	-
	178	-	-	+
	100	+	+	+
	97	+	-	-
	72	-	+	-
B1	4318	+	+	+
	2143	-	-	+
	1378	+	+	+
	990	+	+	+
	733	+	+	+
	570	+	+	+
	348	+	+	+
	294	+	+	+
	255	+	+	+
	145	+	+	+
	86	+	+	+
	74	-	+	-
B7	2500	+	-	+
	1911	-	-	+
	1822	+	-	-
	1200	-	+	-
	1013	+	+	+
	850	+	-	+
	547	+	+	+
	367	+	+	+
	268	+	+	+
	190	-	-	+
	96	-	-	+
	76	-	+	+
	67	+	-	+

Table 6: ISSR scored bands.

ISSR primer	M. s	1	2	3
17898A	631	+	-	-
	500	+	+	+
	350	+	-	-
	150	+	-	-
17898B	974	-	+	+
	665	-	+	-
	300	+	-	+
	80	+	+	+

Table 6: Continued

844A	593	+	+	+
	400	-	+	-
	290	+	+	-
	150	+	+	+
	75	-	+	-
HB10	2200	-	-	+
	1644	-	-	+
	706	+	+	+
	500	+	+	+
	438	+	+	+
	362	-	-	+
	256	-	+	+
	111	+	-	+
	89	-	-	+
	75	-	-	+
HB11	1467	-	-	+
	967	+	+	+
	578	-	-	+
	457	+	+	+
	300	+	+	-
	163	+	+	+
	100	+	+	-
	90	+	+	+
HB13	2083	-	+	+
	1122	-	+	-
	733	+	+	+
	423	-	+	-
	377	+	+	+
	288	+	+	-
	163	+	+	+
	95	+	+	+
	70	+	+	+

Table 7: The number and types of bands and percentage of the total polymorphism in ISSR and RAPD.

		Polymorphic band			
Type	Monomorphic band	unique	shared	Total	Polymorphism%
ISSR	16	15	9	40	60%
RAPD	37	22	16	75	50%

Only four monomorphic and 11 polymorphic bands were recognized in the produced isozyme profiles (Fig. 1 and Table 3). *L. novocanariensis* was distinguished from the other studied species by one unique band. On the other hand, only four shared bands were recorded in the isozyme patterns of all species, four bands were common between *L. azorica* and *L. novocanariensis*, whereas five bands were common between *L. nobilis* and *L. novocanariensis*. *L. azorica* shared six bands with *L. nobilis*. The later taxon was distinguished by seven unique bands. A high level of polymorphism ranging between 50% and 100% (Table 3) were recorded in the three patterns. This reflects a considerable variation among the species under study. The constructed phenogram (Fig. 4a) based on the coding of the produced banding patterns by application of the isozyme technique (15 characters) on the studied taxa of *Laurus* revealed a close relationship between *L. azorica* and *L. novocanariensis*, both taxa were grouped together at the dissimilarity level of 0.99. This was due to their sharing of 4 bands. *L. nobilis* splitted from the other two taxa at the level of 1.57. Max *et al.* [40] when examining the genetic variation based on isozyme analysis in an Alaskan

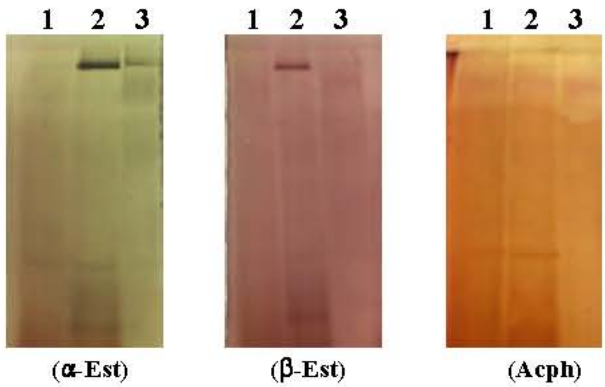

Fig. 1: Electrophoritic banding patterns of α-esterase (α-Est), β-esterase (β-Est) and acid phosphatase (Acph) isozymes for *L. azorica* (1), *L. novocanariensis* (2) and *L. nobilis* (3)

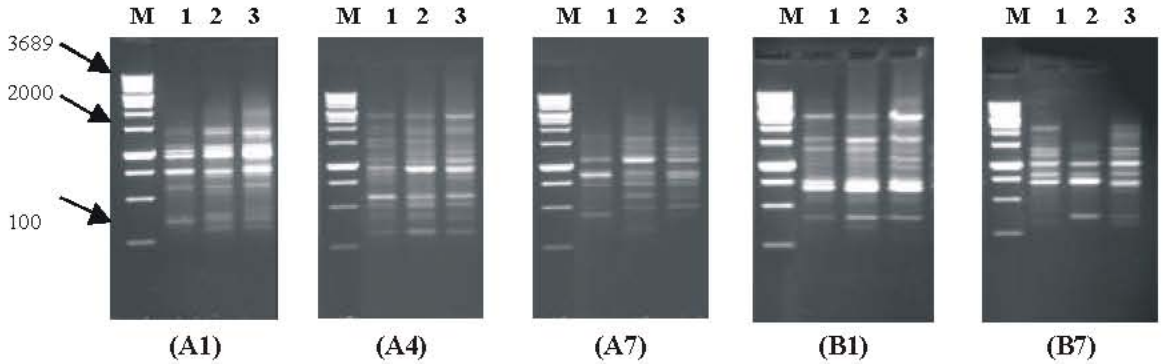

Fig. 2: RAPD profiles of the *Laurus azorica* (1), *Laurus novocanariensis* (2) and *Laurus nobilis* (3) generated by the A1, A4, A7, B1 and B7 primers; M = marker

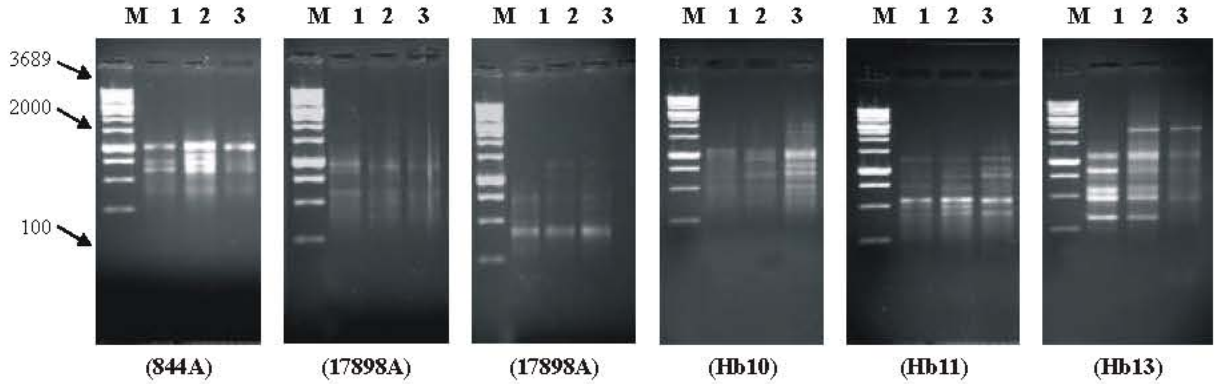

Fig. 3: ISSR profiles of the *Laurus azorica* (1), *Laurus novocanariensis* (2) and *Laurus nobilis* (3) generated by the 844A, 17898A, 17898B, HB10, HB11 and HB13; M = marker

endemic shrub *Dryas octopetala* L. declared that the divergence for a few morphological and life-history characters can occur in response to strong selection, but without divergence at allozyme loci. In another study, Rottenberg *et al*. [41] pointed out that, most of isozyme variation produced, was likely to be as a result of mutational events that alter the performance of their encoding genes. Thus, the sharing of both Macaronesian species of *Laurus* with many bands with the continental *L. nobilis* (six bands shared between *L. azorica* and *L. nobilis* and five bands between *L. novocanariensis* and *L.nobilis*) may give extra supports for the views regarding

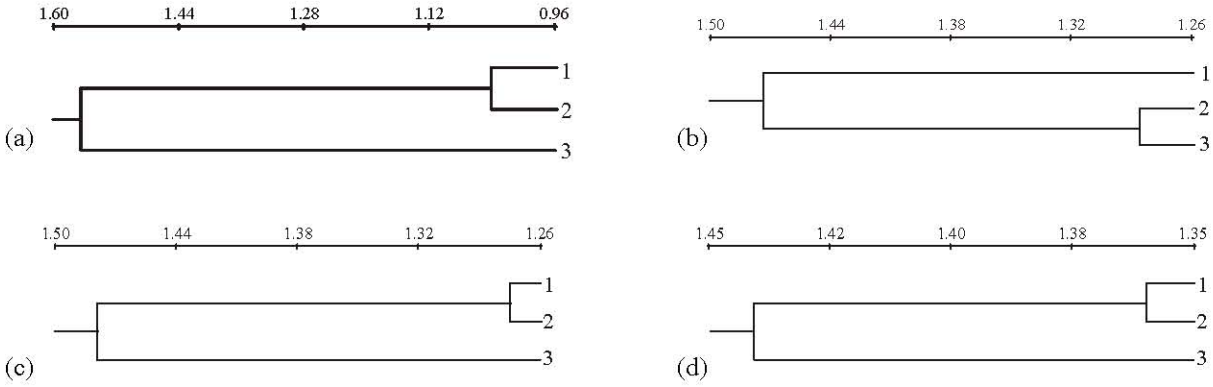

Fig. 4a-d: (a) UPGMA Phenogram of Isozymes (b) UPGMA Phenogram of RAPD
(c) UPGMA Phenogram of ISSR (d) UPGMA Phenogram of Isozymes, RAPD and ISSR

Laurus as a propable monotypic genus [6, 16, 20]. The close similarity in isozyme patterns between the two Macaronesian species of *Laurus* may further put into question the recent splitting of the Madeira and Canary Islands population of *L. azorica* into the species novum (*L. novocanariensis*) as proposed by Rivas Martinez *et al.* [18].

RAPD data produced 75 bands, 37 monomorphic and 38 polymorphic bands (Fig. 2 and Table 5). *L. azorica* was distinguished from the other two species by six unique bands, *L. novocanariensis* by seven unique bands, *L. nobilis* by nine unique bands. On the other hand, only 37 shared bands out of 75 were recorded in the RAPD patterns of all species. The level of polymorphism was 50. The constructed phenogram (Fig. 4b) based on coding of the produced RAPD fragments (utilizing five primers) as 75 binary characters yielded the following: *L. novocanariensis* grouped with *L. nobilis* at the dissimilarity level of 1.29 due to their sharing of 48 bands. *L. nobilis* shared 41 bands with *L. azorica*, while the two-macaronesian laurels (*L. azorica* and *L. novocanariensis* shared only 38 bands. *Laurus azorica* was split from the other two taxa at the level of 1.48. Paul *et al.* [42] stated that high similarities when applying molecular markers could be obtained when selection is applied on the same natural population. Several recent authors addressing the relationships of the Macaronesian archipelago floras have, however, implicitly challenged the Engler [43] and other proponents concept of a unified phytogeographical Macaronesia by classifying the floras of the different archipelagos in different biogeographic regions. For example, Rivas-Martinez *et al.* [44] included the Azores within the Euro-Siberian region and Madeira and the Canaries within the Mediterranean region (Cape Verdes were not considered). On the other hand, Lobin [45] placed the Azores within the sub-Mediterranean subregion, the Canaries and Madeira with western Morocco within a Canarian-Mediterranean subregion. Thus, the two Macaronesian *Laurus* species may have undergone a probable independent recent evolutionary history. RAPD results thus give support to Arroyo Garcia *et al.* [6] who stated that the genetic pattern is indicative of the *Laurus* in Madeira and the Canary Islands (currently *L. novocanariensis*) being derived recently from *L. nobilis*.

Sixteen monomorphic and twenty-four polymorphic bands were recognized in the produced ISSR profiles (Fig. 3 and Table 6). *L azorica* was distinguished from the other two species by three unique bands, *L. novocanariensis* by five unique bands and *L. nobilis* by seven unique bands. On the other hand, only 16 shared bands out of 40 were recorded in the ISSR patterns of all species. High level of polymorphism (60%) was recorded in the three patterns. The constructed phenogram (Fig. 4c) based on the coding of the produced ISSR fragments (utilizing six primers) as 40 binary characters yielded a close relationshipt between *L. azorica* and *L. novocanariensis*, both taxa were grouped together at the dissimilarity level of 1.22. This was due to their sharing of 20 bands. *Laurus nobilis* shared 17 bands with *L. azorica* and 19 bands with *L. novocanariensis*. *L. nobilis* is split from the other two taxa at the level of 1.77. This finding may once more put into question the recent splitting of the Madeira and Canary Islands population of *L. azorica* into the species novum (*L. novocanariensis*).

Tsumura *et al.* [46], Nagaoka and Ogihara [47] reported that either RAPD or ISSR markers are not affected by the environmental conditions and that ISSR markers are more specific than those of RAPD in

determining the genetic variation between taxa. Furthermore, Julie and Pablo [48] reported that the products generated from 5' anchored primers should exhibit more co-dominant polymorphism than RAPD. In the present study, the polymorphism level revealed by ISSR primers was higher than that of RAPD primers as shown in Table 7, which supports such views. The combined data of 130 characters phenogram (Fig. 4d) confirmed the close similarity between the two Macaronesian species of *Laurus*. Both taxa were grouped together at the level of 1.37, whereas the continental *L. nobilis* was split at the level of 1.44. Finally, the study showed a closer relationship between *L. azorica* and *L. novocanariensis* than those between any of them and *L. nobilis*. However, *L. novocanariensis* possessed a higher similarity with the continental *L. nobilis* than *L. azorica*.

ACKNOWLEDGMENT

The author thanks Dr. M.H.A. Loutfy for providing samples of the present study and Dr. M.M. Abou-El-Enain for revising the manuscript.

REFERENCES

1. Mabberley, D.J., 1997. The Plant Book: a Portable Dictionary of the Vascular Plants. Second edition, pp: 393-394.
2. Sfikas, G., 1993. Trees and Shrubs of Greece. Efstathiadis Group (Atenas).
3. Pirone, G., 1995. Alberi, arbusti e liane d'Abruzzo. Cogecstre Edizioni (Penne).
4. Begines, F., 1996. Parque Natural de los Alcornocales, reliquia del terciario. Bio., 8: 7-10.
5. Jalas, J. and J. Suominen, 1991. *Atlas florae Europeae, 9.* Committee for Mapping the Flora Europaea and Societas Biologica Fennica Vanamo. Helsinki University Printer House.
6. Arroyo-García, R., J.M. Martínez-Zapater, J.A. Fernández Prieto and R. Álvarez-Arbesú, 2001. AFLP evaluation of genetic similarity among laurel populations. Euphytica, 122: 155-164.
7. Barbero, M., A. Benabid, C. Peyre and P. Quezel, 1981. Sur la presence au Maroc de *Laurus azorica* (Seub.) Franco. Anales del Jardin botanique de Madrid, 37: 467-472.
8. Bramwell, D.Z., 1983. The endemic flora of the Canary Islands; distribution, relationships and phytogeography. In G. Kunkel [ed.], Biogeography and ecology in the Canary Islands. Dr. W. Junk, The Hague, Netherlands.
9. Ferguson, D.K., 1974. On the taxonomy of recent and fossil species of *Laurus* (Lauraceae). Botanical J. the Linnean Society, 68: 51-72.
10. Loutfy, M.H.A., 2000. On the delimitation of *Laurus* Species (Lauraceae). In proceedings of the 1st Intl. Conference of Biol. Sci., Tanta University (7-8 May 2000), 1: 71-91.
11. Baillon, H., 1872: The natural history of plants. Translated by Hartog, M.M. vol. II London, L. Reeve and Co., 426-480.
12. Moro, R., 1988. Guía de los árboles de España. Editorial Omega.
13. Nees, S.G., 1989. *Laurus*. In: S.H. Walters, A. Brady, C.D. Brickell, J. Cullen, P.S. Green, V.A. Matthews, D.A. Webb, O.F. Yeo and J.C.M. Alexander (Eds.), The European Garden Flora III. Cambridge University Press.
14. Morales, D., M.S. Jimenez, A.M. González-Rodríguez and J. Cermak, 1996. Laurel forest in Tenerife, Canary Island: the site, stand structure and stand leaf distribution. Trees, 11: 34-40.
15. Jiménez, S.M., J. Cermák, J. Kucera and D. Morales, 1996. Laurel Forest in Tenerife, Canary Islands: the annual course of sap flow in *Laurus* trees and stand. J. Hydrobiol., 183: 307-321.
16. Rohwer, J.G., 1993. Lauraceae. In Kubitzki K (ed.): The Families and Genera of Vascular Plants. Springer. Berlin, Heidelberg, New York, pp: 366-391.
17. Bramwell, D. and Z. Bramwell, 1983. Flores silvestres de las Islas Canarias. 2nd Edition. Ed. Rueda.
18. Rivas-Martinez, S., T.E. Diaz, F. Fernández-González, J. Izco, J. Loidi, M. Lousã and A. Penas, 2002. Vascular Plant Communities of Spain and Portugal. Addenda to the Sintaxonomical checklist of 2001, Part II. Itinera Geobotany, 15: 703.
19. Costa, J.C., J. Capelo, R.. Jardim and M. Sequeira, 2004. Catálogo Florístico do Arquipélago da Madeira. Quercetea, 6: 187-200.
20. Rodríguez-Sánchez, F. and J. Arroyo, 2008. Reconstructing the demise of Tethyan plants: climate-driven range dynamics of Laurus since the Pliocene. Global ecol. biogeography, 17: 685-695.
21. Bachmann, L., Y. Rumpler, J.U. Ganzhorn and J. Tomiuk, 2000. Genetic Differentiation among Natural Populations of *Lepilemur ruficaudatus*. Intr. J. Primatology, 21: 853-864.
22. Lienert, J., M. Fischer, J. Schneller and M. Diemer, 2002. Isozyme variability of the wetland specialist *Swertia perennis* (Gentianaceae) in relation to habitat size, isolation and plant fitness. American J. Botany, 89: 801-811.

23. Weeden, N.F. and J.F. Wendel, 1990. *Genetics of plant isozymes*, pp: 46-72. In D.E. Soltis and P.S. Soltis, eds. Isozymes in plant biology. Chapman and Hall, London.
24. Garcia, A. and K. Tsunewaki, 1977. Cytogenetical studies in the genus *Persea* (lauraceae). Electrophoretical studies on peroxidase isozymes. Japanese J. Genetics, 52: 379-38.
25. Moran, G.F., J.C. Bell and S. Prober, 1990. The utility of isozymes in the systematics of some Australian tree groups. Aust. Syst. Bot., 3: 47-57.
26. Moraes, P. and M. Derbyshire, 2002. Estrutura genética de populações naturais de. *Cryptocarya aschersoniana* Mez. (Lauraceae) através de. marcadores isoenzimáticos. Biota neotropica vol 2, no 2, http:// www. biotaneotropica. org. br/ v2n2/ pt/ abstract?article+bn02402022002.
27. Moraes, P. and M. Derbyshire, 2003. Diferenciação genética e diversidade em populações. naturais de *Cryptocarya aschersoniana* Mez. (Lauraceae). Biota neotropica vol 3, no 1, http:// www. biotaneotropica. org.br/v3n1/pt/ abstract?article+bn01803012003.
28. Kantety, R.V., X. Zeng, 1995. Assignment of genetic diversity in dent and popcorn (*Zea mays* L.) inbred lines using intersimple sequence repeat (ISSR) amplification. Molecular Breeding, 1: 365-373.
29. Charters, Y.M., A. Robertson, M.J. Wilkinson and G. Ramsay, 1996. PCR analysis of oilseed rape cultivars (*Brassica napus* L. ssp. *oleifera*) using 5'-anchored simple sequence repeat (SSR) primers. Theoretical and Applied Genetics, 92: 442-447.
30. Tutin, T.G., 1993. *Laurus* in T.G. Tutin *et al.* (eds.), Flora Europaea, 2nd Ed. Vol. 1. Cambridge University Press, pp: 296-297.
31. Short, M., 1994. *Lauraceae*. In I.R. Perss and M.I. Short (ed.). Flora of Madeira. HMSD Publication Centre. London, pp: 100-102.
32. TROPICOS, 2008. Missouri Botanical Garden, http://www.tropicos.org. Accessed on 1. Oct. 2008.
33. IPNI, 2008. The International Plant Names Index. http://www.ipni.org (Accessed 1 October 2008).
34. Stegemann, H., A.M. Afify and K.R. Hussein, 1985. Cultivar identification of dates (*Phoenix dactylifera*) by protein patterns, P.44. 2nd International Symposium of Biochemical Approaches to Identification of cultivars. Brounschweig, Germany.
35. Scandalios, J.C., 1964. Tissue specific isoenzyme variations in maize. J. Heredity, 55: 281-285.
36. Wendel, J.F. and N.F. Weeden, 1989. Visualization and interpretation of plant isozymes. In D.E. Soltis and P.S. Soltis (eds.), Isozymes in plant biol., pp: 5-45. Dioscorides Press, Portland, Oregon, USA.
37. Dellaporta, S.L., J. Wood and J.B. Hicks, 1983. A plant DNA mini preparation. Plant Molecular Biol. Reports, 1: 19-21.
38. Williams, J.G., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey, 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Research, 18: 6531-6535.
39. Rohlf, F.J., 1989. NTSYS-pc, Numerical taxonomy and multivariate analysis system. New York, Exeter pub., Ltd.
40. Max, K.N., S.K. Mouchaty and K.E. Schwaegerle, 1999. Allozyme and morphological variation in two subspecies of *Dryas octopetala* (Rosaceae) in Alaska. American J. Botany, 86:1637.
41. Rottenberg, A., E. Nevo and D. Zohary, 2000. Genetic variability in sexually monomorphic and dimorphic populations of *Populus euphratica* (Salicaceae). Can. J. Forest. Res., 30: 482-486.
42. Paul, S., F.N. Wachira, W. Powell and R. Waugh, 1997. Diversity and genetic differentiation among populations of Indian and Kenyan Tea (*Camellia sinensis* (L.) O. Kuntze) revealed by AFLP markers. Theoretical and Applied Genetics, 94: 255-263.
43. Engler, A., 1879. Versuch einer Eintwicklugsgeschichte, insbesondere der Florengebiete seit der Tertiärperiode. I. Die extra-tropischen Gebiete der nördlischen Hemisphäre. Engelmann, Leipzig, Germany.
44. Rivas-Martínez, S., A. Penas and T.E. Diaz, 2004. Biogeographic map of Europe. Cartographic Service. University of Leon, Spain. http:// www. globalbioclimatics.org/form/maps.htm.
45. Lobin, W., 1982. Untersuchung über Flora, Vegetation und biogeographische Beziehungen der Kapverdischen Inseln. Courier Forschungsinstitut Senckenberg, 53: 1-112.
46. Tsumura, Y., K. Ohba and S.H. Strauss, 1996. Diversity and inheritance of intersimple sequence repeat polymorphisms in Douglas-fir (*Pseudotsuga menziesii*) and sugi (*Cryptomeria japonica*). Theoretical and Applied Genetics, 92: 40-45.
47. Nagaoka, T. and Y. Ogihara, 1997. Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. Theoretical and Applied Genetics, 94: 597-602.
48. Julie, M.V. and A.S. Pablo, 1997. Direct amplification from micro-satellites, detection of simple sequence repeat-based polymorphisms without cloning, pp: 137-150. In G. Caetano-Anolles and P.M. Gresshof (eds) DNA Markers, Protocols, Applications and Overviews, Wiely-Liss, Inc.