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INTRODUCTION

Two complementary projects, based at Bath University and HRI Wellesbourne, are attempting to introduce resistance or tolerance to the two main destructive diseases of commercial watercress; crook-root disease (Tomlinson, 1958) and watercress cholorotic leaf spot virus (Walsh, 1989). The projects make use of two different approaches to select improved disease resistant watercress plants; a biotechnological approach producing genetically altered commercial watercress plants via *in vitro* somaclonal culture at Bath (J. Claxton) and a traditional approach at Wellesbourne (J. Walsh) of hybridising commercial watercress plants with other watercress and related crucifers species which have been shown to have some resistance to watercress diseases.

Both approaches to improving commercial watercress strains are centred on selecting and improving disease resistance inherent within the present watercress crop plants or introducing it from related species. To date, no disease resistance has been identified from any commercial plant lines (J. Walsh and J. Claxton, per comms) and limited disease resistance has been identified in plants believed to be closely related to watercress (J. Walsh). Neither have any hybrids been formed between these disease resistant related crucifer species and watercress. The central aim of the project was firstly, to measure the levels of heterogeneity or amount of genetic variation within the commercial watercress population and then to measure the genetic distance between watercress and those Brassicaceae species which have disease resistance characters and are believed to be closely related to watercress. The results from both these studies complemented and have aided both projects improving disease resistance in watercress.

1 Genetic Variation within Selected Commercial Watercress Lines

A molecular technique was selected over a phenotypic or protein-based assay to measure genetic diversity between commercial watercress populations. This would enable an impartial and accurate comparison between an impartial and accurate comparison between individuals disregarding any environmental or selective constraints which might be inherent between the different farms.

Commercial Watercress Populations Sampled

Leaf and stalk material were collected from 20 randomly chosen plants growing in one bed on each of the ten commercial sites listed in the table below. Sampling took place in early February 1994, over a two day period. Vegetative material was collected into plastic bags and then later washed in the laboratory and stored at -20°C until processed.

Table 1. Location and name of watercress farms from which samples were collected

Site	Grower and location of farm	River	Grid ref.	No
1	Mr. TA Tyler, Simar, Dimmocks, Sarrat Fm, Rickmansworth, Herts	Chess	027992 (176)	1
	As above	-"-	-"-	2
2	Vitacress Salads, St Mary Bourne, nr Whitchurch, Hants	Bourne	429507 (185)	3
3	Vitacress Salads, Warnford, nr Alresford, Hants	Meon	625249 (185)	4
4	Vitacress Salads, Fob Down fm, Old Alresford, Hants	Itchen	572341 (185)	5
5	Mr. C. Barter, B&M watercress, Bighton, nr Alresford, Hants	Itchen	582307 (185)	6
6	Mr. NT Barter, Bishopstone, nr Salisbury, Wilts ♦	Test	352340 (183)	7
7	Mr. K. Hitchings, Chalk Valley Watercress, nr Bishopstone, Salisbury, Wilts	Test	327285 (183)	8
8	Vitacress Salads, Diddings Farm, Bere Regis, Dorset	Piddle or Trent	853944 (194)	9
9	Mr. TW. Jesty, Sylvasprings Farm, Blandford Forum, Dorset	Frome	665882 (194)	10
10	Mr JHW. Hurd, Hill Deverill, nr Warminster, Wilts	Wylye	869417 (183)	11

♦ Indicates a certified Organic Grower (at time of collection) who did not use pesticides or Zinc to control crook-root on his farm

♦♦ The numbers in parenthesis refer to the O.S. map no. Landranger series 1:50,000.

Vegetative material was collected from two different watercress beds from Mr. Tyler's farm, Sarrat, Herts. (site numbered 1). Sample numbered 2 was collected from a bed which had been continually cultivated using the same vegetative material for the previous twenty years.

Molecular Assessment of Genetic Variation Between Commercial Watercress Farms

A PCR based fingerprinting method, RAPD-PCR (Random Amplified Polymorphic DNA; Williams *et al.*, 1990) was the molecular method used as this allows quick and accurate fingerprinting of a potentially large number of individuals without having prior information of each genome. RAPD-PCR parameters and conditions were optimised to ensure reproducible and reliable analysis of watercress genomes before the watercress samples from the farms listed in table 1.

Watercress RAPD-PCR protocol

Watercress DNA for RAPD-PCR analysis was isolated using an adapted CTAB based protocol (Dellaporta *et al.*, 1993). The following thermocycler protocol was the optimised regime used to generate RAPD-PCR products from watercress DNA. Reactions were carried out using a Hybaid Omigene™ Thermocycler maintained at ambient temperature.

95°C	5 min.	
34°C	30 sec	
	increasing at 3°C/sec ramp to 50°C	1 cycle
72°C	30 sec	
94°C	30 sec	
36°C	30 sec	
	increasing at 3°C/sec ramp to 50°C	20 cycles
72°C	30 sec	
94°C	5 sec	
36°C	30 sec	
	increasing at 3°C/sec ramp to 50°C	10 cycles
72°C	30 sec 1 sec/cycle extension	
72°C	5 min.	1 cycle

Each PCR reaction was carried out in a final volume of 25 µl containing 4 mM $MgCl_2$, 1 x PCR reaction buffer (50 mM KCl and 10 mM Tris-HCl (pH 8.3)), 0.2 mM of each dNTP and 0.05% Glycerol. When setting up a PCR, a 'master mix' was made containing enough reaction components and (either 10 or 20 picomols) 10-mer for the total number of reactions being set-up. This 'master mix' was gently vortexed and dispensed into 0.5 ml eppendorf tubes, spun down briefly in a centrifuge and then overlaid with a drop of mineral oil (Sigma). The closed tubes were then placed under short-wave (320 nm) UV light for 10 min before 20 ng template DNA was added to each tube, pippetting the DNA under the oil layer. The tubes were placed in the thermocycler, the reaction started and allowed to run for 4.5 min. After 4.5 min at 95°C, the thermocycler program was paused, the tubes were removed and placed immediately on ice for at least 1 min, 1 unit *Taq* DNA polymerase (Promega) was added to each tube (again pippetting it under the oil layer), then the tubes were returned to the thermocycler at 95°C and the PCR thermocycler program was continued.

RAPD-PCR Assay procedure

DNA was isolated from each commercial watercress population sample, grinding all the leaf material in a large pestle and mortar and using all the material for isolation. DNA was quantified by comparison to known standard molecular weight markers and an aliquot stored at -20°C. The remaining DNA was diluted to a final concentration of 5 ng μl^{-1} and stored at 4°C. A 'master mix' of optimised PCR ingredients was made up and a RAPD-PCR reaction set-up as detailed previously. In each PCR, all 11 commercial watercress samples, 2 control plant samples, sweet potato (*Ipomoea batatas*) and Japanese watercress and a negative control reaction (PCR mix with no DNA) were always amplified. Chinese cabbage and sweet potato were included as 'outgroup' species or control species that are distantly related to watercress.

Method of Recording and Analysis of RAPD-PCR Results

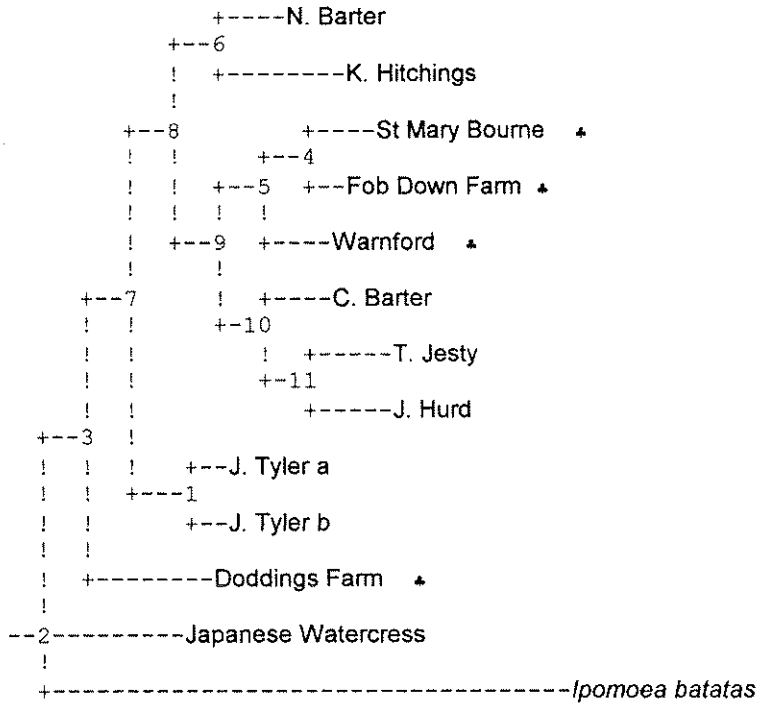
Each RAPD reaction was repeated three times with each primer to ensure reproducibility between successive PCR runs. Each samples' fingerprint for each of the 17 different primers used was visually compared (using the 35mm photographs taken for each RAPD-PCR gel) and only those amplification bands that appeared consistently in all three separate reactions were included in the final analysis. A total of 157 RAPD-PCR amplification products were used to draw dendrograms of relatedness 49 (28%) were polymorphic amongst the watercress populations.

Amplification products of the same size (as compared to a 100 bp ladder) were scored as being either absent or present (recorded using either 0 or 1 in binary code) for each of the samples analysed (otherwise known as an Operational Taxonomic Unit or OTU). This binary coding score was recorded in tabular form (binary matrix) for each OTU and these similarity matrices were condensed into distance matrices using two different coefficients; the Jaccard (1901) and Simple-Matching (Sokal and Michener, 1958) coefficients where each uses an algorithm placing different emphasis on the absence or presence of bands (or the arrangement of 1 and 0's) between any two OTU's in the data matrix.

Once distance matrices were calculated, the relationships between all the OTU's were represented graphically by dendrograms using both Neighbor-Joining and UPGMA (Unweighted Pair-Group Method using an Arithmetic average) (Sneath and Sokal, 1973) analysis programs in the distance program NEIGHBOR within the PHYLIP computer package (Felsenstein, 1989).

Fig 1. overleaf is one such dendrogram where most closely related OTU's are placed close to each other at distances (given in arbitary units) given in the table below the dendrogram.

Fig. 1 Dendrogram showing relationship between commercial watercress drawn by the Neighbor-Joining method with similarity data calculated with the Jaccard coefficient.



♣ Indicates Vitacress Salad farms

Between	And	Length	Between	And	Length
2	3	0.0318	3	7	0.01850
7	8	0.01941	8	6	0.02331
6	N. Barter	0.09053	6	K. Hitchings	0.15734
8	9	0.00531	9	5	0.02804
5	4	0.00611	4	St Mary Bourne ♣	0.09605
4	Fob Down ♣	0.06136	5	Warnford ♣	0.08472
9	10	0.01321	10	C Barter	0.07921
10	11	0.00421	11	T Jesty	0.10090
11	J Hurd	0.10093	7	1	0.07300
1	J Tyler a	0.04957	1	J Tyler b	0.04134
3	Doddings ♣	0.15541	2	Japanese	0.17500
2	Sweet potato	0.60549			

The RAPD-PCR technique was sensitive enough to distinguish between individual samples as shown by fig. 1 where an expected pattern of diversity amongst the different farms placing two samples from one farm (J Tyler) close to each other and clustering farms which use the same seed material (Vitacress Salads Ltd) but are physically distant to each other (St Mary Bourne, Fob Down and Warnford) is seen. Although some anomalies were apparent by RAPD-PCR analysis (viz. the fourth Vitacress farm, Diddings Farm, not clustering with other Vitacress Farms) the different methods of data analysis showed a consistent pattern where the outgroup species (sweet potato) was placed at a great distance from watercress; the uncultivated

Japanese watercress variety was always placed away from the commercial cresses and they themselves were placed very close together.

The RAPD-PCR study showed that commercial watercress is a heterogeneous population but the genetic distances between the individual commercial populations examined is very small as seen by the length figures on the dendrogram where the distance between the two samples from J Tylers' and J Jesty and J Hurds' farms are represented by 5 and 9 polymorphic RAPD-PCR bands respectively. The low levels of genetic variation measured by RAPD-PCR analysis highlight the difficulty and failure, to date, of selecting and then improving on disease tolerant or resistant watercress from the commercial populations.

Genetic Diversity between Watercress and Related Crucifers

The survey of genetic variation was extended to examine diversity between wild watercress plants and also between watercress and 'closely' related Brassicaceae species which showed resistance to the watercress disease (J Walsh, Watercress Association AGM 1993 & 1994). Wild watercress were collected from rivers and streams throughout the British Isles and related Brassicaceae species donated by J Walsh; details of all plants assessed are given on Table 2 overleaf.

Table 2. Watercress and Related Brassiceae Species analysed by RAPD-PCR and restriction enzyme analysis of rDNA gene

Related Species	Common Name and Origin
Cardamine flexuosa ♠♦	Wavy Bittercress (J Walsh, Wellesbourne)
Cardamine hirsuta ♠♦	Hairy Bittercress (J Walsh, Wellesbourne)
Lepidium sativum ♠♦	Curly cress (Sutton Seeds)
Barbarea vulgaris ♦	Landcress (Sutton Seeds)
Brassica oleraceae var. chinensis ♠♦	Chinese cabbage (Sutton Seeds)
Barbarea verna ♠	American landcress (Sutton Seeds)
Rorippa heterophylla ♠♦	'Snotplant' (D Blakesley, Bath University)
Rorippa palustris ♠♦	Yellowcress (J Walsh, HRI Wellesbourne)
Rorippa sylvestris ♠♦	Yellowcress (J Walsh, HRI Wellesbourne)

Wild watercress	Common Name and Origin
Watercress USA 1 ♠♦	Florida, USA (S Rothwell)
Watercress USA 1M ♠♦	Florida, USA (S Rothwell)
Watercress USA 1U ♠♦	Florida, USA (S Rothwell)
Watercress USA WS ♠♦	Florida, USA (S Rothwell)
Watercress Control ♠♦	Vitacress Salads (S Rothwell)
Japan ♠	(J Walsh, HRI Wellesbourne)
New Zealand ♠	(J Walsh, HRI Wellesbourne)
France ♠	(J Walsh, HRI Wellesbourne)
Italy ♠	(J Walsh, HRI Wellesbourne)
Spain ♠	(J Walsh, HRI Wellesbourne)
Ballyogan ♠	Inniscrone, Co. Mayo, Eire (G Sheridan)
Caracardin ♠	Inniscrone, Co. Mayo, Eire (G Sheridan)
Blackburn ♠	Lancashire (L Smithson)
Hickington ♠	Suffolk (J Claxton)
Wookey Hole ♠	Cheddar, Somerset (J Claxton)
Kingston Deverill ♠	Warminster, Wiltshire (Sheridan and Claxton)
Salisbury ♠	Wiltshire (L Smithson)

Two different but completely molecular techniques were employed; RAPD-PCR (♦) and restriction enzyme analysis of the nuclear rDNA gene (♠) and the plants analysed by each technique are marked as such in the table.

RAPD-PCR analysis was carried using the optimised protocol and data analysis methods as previously mentioned and fig 2. shows a dendrogram of relationships between all plant species considered. Restriction analysis of the rDNA gene focuses on genetic variation within a specific region which contains conserved codegenic areas and less conserved non-coding regions (Hamby & Zimmer, 1989) unlike RAPD-PCR which examines random regions of the entire plant genome. Comparison of a specific gene is more appropriate when examining species variation. PCR primers were designed after Sun *et al.*, (1993) which amplified a 850 bp region encoding the entire 5.8S gene and two non-protein coding spacers, ITS1 and ITS2. This region was amplified from the plant species and subsequently digested with specific restriction endonucleases. Digested PCR fragments were detected by electrophoresis and polymorphism between the species noted by absence and presence of sized DNA products and analysed using the same techniques as with RAPD-PCR

analysis. Fig 3. illustrates the relationship between all the species examined using RFLP analysis of the nuclear rDNA gene.

The two different molecular methods of analysis showed a complementary pattern of relationships both between and within species. Individuals of the same genus were all clustered together (viz. *Cardamine* species) except for the surprising position of all the watercress (*Rorippa nasturtium-aquaticum*) clustered distant to other *Rorippa* species (viz. *R. sylvestris, R. heterophylla* and *R. palustris*) with which it is taxonomically classified. Indeed commercial ('Watercress Control' on figs 2 & 3) and wild watercresses were placed at an equal distance to all other species with which is was compared by RFLP analysis of the rDNA gene.

Fig 2. Dendrogram of relationship between Commercial Watercress and Related *Brassicaceae* determined by RAPD-PCR analysis

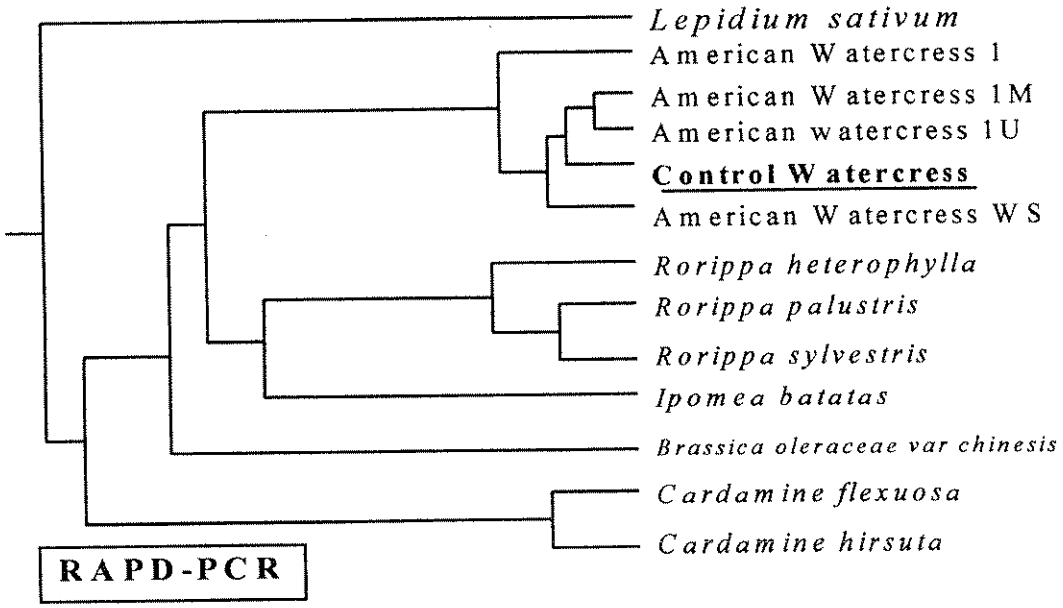
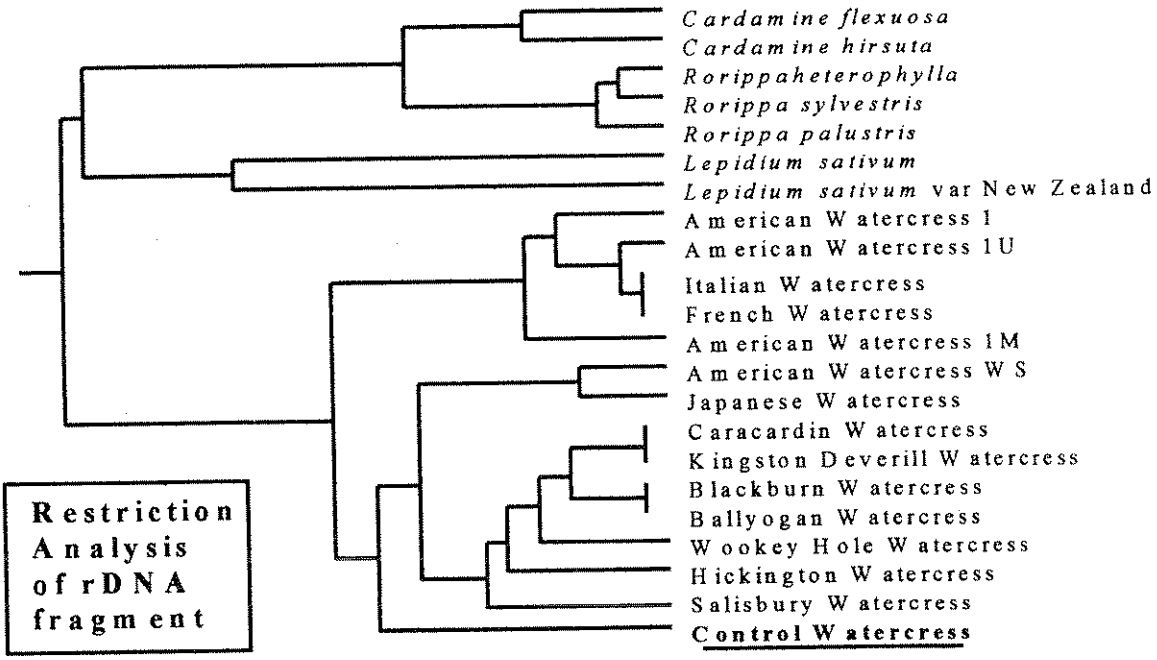


Fig 3. Dendrogram showing relationship between Wild and Commercial Watercress and Related *Brassicaceae* using RFLP analysis of rDNA gene.

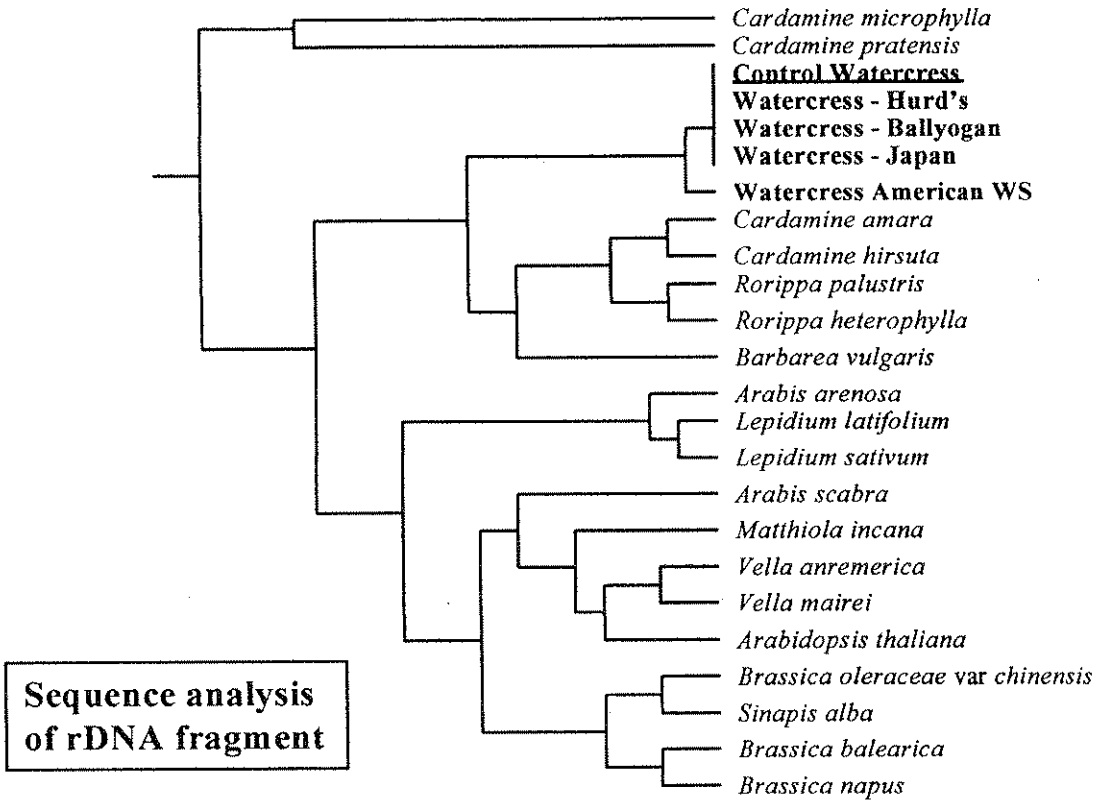


These results throw some doubt on the current taxonomic classification of watercress in the *Rorippa* gene. They also throw some light on the difficulty and failure to date (Howard & Manton, 1948; Walsh, per Comm. 1995) of choosing an ideal 'closely related' Brassicaceae species with which to hybridise with watercress based solely on morphological characters. This suggests that watercress is perhaps not as related to the selected *Cardamine* and other *Rorippa* species as we thought and that maybe more genetically close species, which have not yet been considered for use in improving disease resistance to watercress, exist within the Brassicaceae.

3. Measure of Genetic Distance between Watercress and Other Brassicaceae by rDNA Sequence Analysis

The rDNA gene fragment (5.8S and two spacer regions) was manually sequenced from commercial watercress and Chinese cabbage to assess the relative heterozygisity and hence usefulness of this gene region at measuring the genetic distance between watercress and other *Brassicaceae* species. This initial control analysis showed that it was a suitable region of genomic DNA with which to compare and ensure relatedness at the levels proposed (viz. genera, species and individuals). In collaboration with the Molecular Systematics Laboratory, Royal Botanic Gardens Kew, the rDNA fragment of eighteen other species (all *Rorippa, Cardamine, Barbarea*, and *Arabis* species) were sequenced automatically using individual plants from Kew Gardens Living and Germplasm Collections. Sequence data from eight other related taxa were supplied by Kew gardens for use as outgroups and the relationship between all twenty-eight taxa is shown in fig 4.

Fig. 4 Dendrogram showing relationship between Watercress and Other *Brassicaceae* Species determined by Sequence Analysis of the nuclear rDNA gene



With one or two exceptions (viz. *Cardamine*) all species of the same genus were clustered close together supporting both the method of sequence data analysis the current phenotypically-based taxonomic identification and classification scheme. The dendrogram showed that the watercress lines were practically identical to each other within the rDNA gene region and, as with previous RAPD-PCR and RFLP analysis of the rDNA gene, are distant to all other *Rorippa* species with which it is currently classified. Watercress is placed equally related to the other *Rorippa* species (*R. heterophylla* and *R. palustris*), *Cardamine amara* and *C. hirsuta* and *Barbarea vulgaris* (a wintercress), a species not yet considered for hybridisation with watercress to improve disease resistance.

4. Concluding Remarks

The molecular analysis of genetic variation between watercress and related Brassicaceae species has shown that

- There is a degree of genetic variation within commercial and wild watercress varieties but the extent of variation is very small, supporting the view that a disease resistant line is unlikely to be found amongst these populations
- From sequence analysis *Barbarea vulgaris* (and possibly other species of this genus) is of an equal distance to watercress as other *Rorippa* and *Cardamine* species, and suggests that *Barbarea* species should also be considered in a traditional breeding program.
- From the sequence analysis of the ITS region that the present classification of watercress as *Rorippa nasturtium-aquaticum* should be redefined to illustrate the difference, (morphologically and) genetically from all the other *Rorippas* species.

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