Transmission des génomes cytoplasmiques et phylogénie moléculaire chez Actinidia

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Transmission of cytoplasmic genomes and molecular phylogeny in *Actinidia*

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Résumé

Le genre *Actinidia* (kiwi) est endémique de Chine et de domestication récente. La classification, controversée, et l'histoire évolutive, mal connue, justifient que soient approfondies les relations phylogénétiques entre les espèces qui le composent. *Actinidia* est un genre de plantes du groupe des angiospermes connu pour le mode d'hérédité contrasté de ses génomes chloroplastique (cp) et mitochondrial (mt). Le mode d'hérédité paternelle de l'ADNcp, précédemment démontré à l'échelle interspécifique, est confirmé ici à l'échelle intraspécifique. La découverte et l'étude d'une chimère plastidique nous conduisent à conclure que de rares cas de transmission biparentale peuvent se produire. Nous avons aussi mis en évidence un découplage dans la transmission des génomes cp et nucléaire lors de la gynogenèse, ce qui constitue un scénario alternatif au modèle d'hybridation/introgression communément invoqué pour expliquer une capture chloroplastique. Les deux génomes cytoplasmiques ont été utilisés pour inférer l'histoire évolutive des *Actinidia*. La détection d'incongruences entre les phylogénies cp et mt confirme quelques-uns unes des réticulations déjà mises en relief par la phylogénie de gènes nucléaires et identifie d'autres événements d'hybridation. Les données cp et mt ne permettent cependant pas de détecter toutes les hybridations. Cela peut être dû à l'origine multiple des polyploïdes, comme nous l'avons montré pour un taxon particulier. Globalement, l'information tirée des trois génomes indique qu'hybridation et polyploïdisation sont fréquentes chez *Actinidia*. Nous confirmons une origine autopolyploïde chez les deux espèces cultivées *A. chinensis* et *A. deliciosa*, et, sur la base de notre échantillon, nous évaluons à au moins un quart les taxons d'*Actinidia* possédant une origine hybride, caractéristique qui peut expliquer la confusion taxonomique souvent signalée pour ce genre.

Mots-clés : *Actinidia*, capture chloroplastique, hérédité chloroplastique paternelle, hybridation, génomes cytoplasmiques, incongruence, kiwi, phylogénie moléculaire, polyploïdisation, spéciation réticulée, chloroplaste, mitochondrie.

Abstract

Actinidia genus (kiwifruit) is endemic to China and has been domesticated recently. The classification, debated, and the evolutionary history, little known, justify to investigate the phylogenetic relationships between the species. *Actinidia* is an angiosperm plant genus known for the contrasted mode of inheritance of its chloroplast (cp) and mitochondrial (mt) genomes. The paternal mode of inheritance of cpDNA previously demonstrated at the interspecific level is confirmed here at the intraspecific level. The finding and the study of one case of plastid chimerism lead us to conclude that rare cases of biparental transmission can occur. We have also demonstrated an uncoupling of the transmission of the chloroplast and nuclear genomes occurring during gynogenesis, which represents an alternative scenario to the hybridization/introgression model commonly invoked to account for a chloroplast capture. The two cytoplasmic genomes were used to infer the evolutionary history of *Actinidia.* The detection of incongruences between the cp and mt phylogenies confirms some of the reticulations first emphasized from a nuclear gene phylogeny and diagnoses other hybridization events. However, the cp and mt data do not allow the detection of all the hybridizations. This could be due to the multiple origin of the polyploids, as we have documented for one particular taxon. Finally, the information from the three genomes indicate that hybridization and polyploidization are common in *Actinidia*. We confirmed the autopolyploid origin in the two cultivated species *A. chinensis* and *A. deliciosa*, and, on the basis of our sampling, we evaluate that at least one fourth of the *Actinidia* taxa are of hybrid origin, a feature that could account for the taxonomic confusion often reported for that genus.

Keywords: *Actinidia*, chloroplast capture, chloroplast paternal inheritance, cytoplasmic genomes, hybridization, kiwifruit, incongruence, molecular phylogeny, polyploidization, reticulate speciation, chloroplast, mitochondrion.

Sommaire

Avant-propos

J'ai débuté ce travail de thèse sur *Actinidia* fin 1999 après avoir passé dix ans à animer un programme d'amélioration génétique sur le kiwi et les espèces apparentées. Cet "actif " m'a permis d'esquisser un sujet de thèse. L'expérience du praticien et l'aide bienveillante et efficace de mon directeur de thèse et des membres du comité de thèse m'ont aidée à accomplir cet effort de longue haleine. La connaissance du matériel végétal et les collaborations nationales et internationales m'ont permis d'accéder à la diversité génétique et de trouver le financement nécessaire au coûteux travail de laboratoire.

Ma volonté est d'apporter une réflexion et des résultats scientifiques sur ce genre mal connu du groupe des angiospermes. Cette méconnaissance résulte de l'histoire propre à ce genre endémique de régions du monde restées longtemps difficiles d'accès. La domestication du kiwi est récente même si des documents de la Chine ancienne témoignent que ses fruits faisaient déjà l'objet de cueillette en forêt sous la Dynastie des West-Han, soit 200 ans avant JC (Cui *et al.* 2002). La culture a seulement commencé au milieu du XX^{e} siècle sous l'impulsion de pépiniéristes néo-zélandais.

Le non-initié trouvera ci-après une petite liste d'espèces qu'il est susceptible de trouver en Europe, sur les étals ou dans les jardins :

- *A. deliciosa*, espèce la plus cultivée dans le monde et communément connue sous le nom de kiwi ou kiwifruit.
- *A. chinensis*, espèce proche de la première, dont une variété à chair jaune est apparue sur nos étals en 2000.
- *A. arguta*, une espèce restée confidentielle qui fournit des petits fruits verts ou rouges, lisses, de la taille d'une prune, baptisés kiwaïs par un pépiniériste avant-gardiste français dans les années 80, et baby kiwis dans le reste de l'Europe. Les quelques hectares de vergers existant en Europe produisent en septembre.
- *A. kolomikta*, une espèce anciennement introduite en Europe qui trouve sa place dans les jardins botaniques grâce à la qualité ornementale de son feuillage panaché blanc et rouge.

Le genre *Actinidia* comprend un grand nombre d'espèces, dont certaines ne sont connues que de quelques rares spécialistes chinois. Pour beaucoup d'entre-elles, aucun spécimen vivant n'a pu franchir les frontières de la Chine car elles font toutes l'objet d'une interdiction de sortie du territoire. Les autorités du pays souhaitent ainsi protéger l'accès aux ressources génétiques. Comme en témoignent les

nombreuses descriptions de nouveaux taxons effectuées par les botanistes chinois et, consécutivement, l'allongement de la liste des espèces à chaque nouvelle révision de la classification, la connaissance de la diversité qu'offre ce genre reste encore incomplète. Parallèlement, certaines espèces décrites lors de précédentes prospections semblent avoir disparu des milieux naturels. Dès les années 90, la priorité des autorités chinoises était de mieux connaître le patrimoine national, de raisonner la conservation des ressources génétiques et de valoriser de nouvelles variétés fruitières. Dans ce contexte favorable, un projet européen ayant pour objet les ressources génétiques *Actinidia* et réunissant plusieurs équipes européennes et chinoises a vu le jour. Ce projet a joué pour moi le rôle de catalyseur.

Parmi les nombreuses pistes qui se sont offertes à moi, j'ai décidé de concentrer mes efforts sur la reconstruction de l'histoire évolutive du genre *Actinidia* sur la base des données moléculaires fournies par les génomes chloroplastique et mitochondrial. Le présent mémoire est constitué d'une introduction qui décrit le but et la démarche du phylogénéticien. Cette introduction comprend un certain nombre de concepts et définitions de termes utilisés en phylogénie. La connaissance du mode d'hérédité des génomes cytoplasmiques constituait un préalable à leur utilisation comme sources d'informations phylogénétiques. Nous n'avons cependant pas dédaigné les "digressions" riches en enseignement et finalement proches de nos préoccupations premières : ce fut le cas quand nous fûmes confrontés à un cas inhabituel d'*hétéroplasmie* puis de *capture chloroplastique*, termes qui seront précisés plus loin.

Les résultats obtenus ont été assemblés dans ma thèse en quatre parties distinctes qui ont déjà ou feront très prochainement l'objet de publications. Chaque partie est constituée d'un résumé, d'un préambule, tous deux rédigés en français, du corps de l'article en anglais, et de commentaires critiques. Pour chacune, j'ai jugé intéressant d'y adjoindre une présentation sommaire des autres pistes de recherches alors envisagées, délaissées du fait de contingences matérielles (manque de temps, matériel végétal indisponible, techniques non maîtrisées) mais toujours d'actualité. Dans la quatrième partie, l'importance des résultats justifiait la valorisation en trois articles distincts. Un article, déjà rédigé, sera soumis dès que l'herbier et les séquences qui s'y rapportent seront déposés, les deux autres sont en préparation et font l'objet d'un sous-chapitre intitulé "compléments de discussion". Enfin, un article concernant les échecs/réussites d'hybridations expérimentales conduites entre diverses espèces d'*Actinidia* est paru dans le même temps. L'objectif principal de cet article est de détailler les techniques mises en œuvre pour obtenir le sauvetage d'embryons et d'en comparer l'efficacité. Les résultats obtenus sont par trop fragmentaires pour tirer des conclusions claires sur l'interfécondité des espèces, sujet en rapport avec les préoccupations du phylogénéticien. Cet article a donc été relégué en Annexe.

La littérature phylogénétique comporte de nombreux concepts, notions et termes pouvant sembler hermétiques. Pour faciliter le repérage du lecteur, ils sont indiqués en italique chaque fois qu'ils sont utilisés pour la première fois dans le document et un index les récapitule à la fin du document

Introduction

Le premier système de nomenclature hiérarchique décrivant la diversité du monde vivant a été développé par Linné en 1735. Différents systèmes de classification se sont ensuite succédés, imprégnés des idées religieuses et philosophiques de leur époque. Les premiers s'attachaient à proposer un système logique de classement des objets en catégories sans chercher à rendre compte des liens de filiation entre les organismes. L'*espèce*, subdivision ultime des classifications, recouvre plusieurs définitions selon le critère retenu : ressemblance, capacité à s'hybrider, ascendance commune… Afin de limiter les confusions, Grant (1981) conseille de ne pas utiliser le terme seul, mais de lui adjoindre un qualificatif. Parmi les 22 concepts d'espèces comptabilisés par Mayden (1997), on citera :

- *espèce taxonomique* ou *morpho-espèce* : Partant du constat, ou du principe, que l'aspect extérieur des organismes vivants varie de manière discontinue, les systématiciens ont défini l'espèce sur la base de caractères morphologiques. "*Species are the smallest groups that are consistently and persistently distinct, and distinguishable by ordinary means*" (Cronquist 1978).
- *espèce biologique* : La définition la plus utilisée et la moins controversée reste celle de Mayr (1963) qui circonscrit les espèces à des "*groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups*". Ce concept d'espèce biologique est cependant source d'ambiguïté pour les végétaux car toutes les espèces de plantes ne sont pas reproductivement isolées. La spéciation par hybridation est d'ailleurs souvent invoquée par les botanistes pour rendre compte d'espèces morphologiquement intermédiaires (Rieseberg 1997).
- *espèce phylogénétique* : Sous l'impulsion des phylogénéticiens, le besoin s'est fait sentir d'intégrer à la notion d'espèce une dimension généalogique qu'elle n'avait pas jusqu'alors (Claridge *et al.* 1997). La définition d'espèce phylogénétique la plus citée est celle de Cracraft (1983) : "*smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent*".

La phylogénie

Le terme *phylogénie* apparaît au milieu du XIXe siècle pour définir la succession des espèces animales et végétales au cours du temps (Haeckel 1866). Une définition plus précise en est donnée par Charles Darwin, il introduit le critère de filiation comme base de la phylogénie (Darwin 1859). Trois disciplines, l'anatomie comparée, l'ontogénie et la paléontologie, ont fourni le cadre des études phylogénétiques dans le passé, désormais rejointes par la génétique moléculaire. Aujourd'hui, de gros efforts sont fournis par les systématiciens pour réviser les classifications afin de mieux respecter les généalogies.

Les principes de construction phylogénétiques

Quelle que soit la discipline, la reconstruction phylogénétique à partir d'un jeu de données repose sur le concept de ressemblance. Les règles différent selon les écoles (Darlu et Tassy 1993). Pour le phylogénéticien, le concept de similitude, ou ressemblance, peut être divisé en *homologie* (similitude héritée d'un ancêtre commun) et *homoplasie* (similitude sans rapport avec la filiation). L'homoplasie se subdivise elle-même en *convergence* (apparition indépendante d'un nouveau caractère chez deux espèces) et *réversion* (acquisition d'un caractère ayant l'apparence de la forme ancestrale). Un caractère peut prendre plusieurs états : l'état primitif ou ancestral (*plésiomorphe*) et l'état dérivé (*apomorphe*). Une *autapomorphie* est une apomorphie présente chez un seul taxon, par opposition à une *synapomorphie* qui est une apomorphie partagée par plusieurs taxons. Les systématiciens phénéticiens se fondent sur le concept de *similitude globale*, exploitant les homologies et les homoplasies sans distinction. Pour les systématiciens partisans de l'école *cladiste*, seul le partage par différentes entités d'états apomorphes est signe d'une parenté (Hennig 1950). Le chemin évolutif privilégié par le cladisticien est celui qui minimise le nombre de caractères homoplasiques (principe de *parcimonie*), et donc le nombre de changements d'états. Enfin, les *approches probabilistes* diffèrent des deux précédentes en ce qu'elles reposent sur l'élaboration d'un modèle d'évolution des caractères défini *a priori*. Le modèle et les observations servent ensuite à calculer une probabilité associée à chaque chemin possible, le plus vraisemblable étant celui qui est finalement retenu pour illustrer l'histoire évolutive du groupe. Cette approche n'ayant véritablement de sens que pour les données moléculaires (établir un modèle d'évolution des caractères morphologiques *a priori* paraît illusoire à ce jour), son développement est un peu plus récent. On trouve une description détaillée et critique de ces trois méthodes de reconstruction dans la littérature (p. ex. Felsenstein 1988; Darlu et Tassy 1993; Doyle et Gaut 2000).

Les sources d'informations phylogénétiques

La reconstruction de l'histoire évolutive d'un groupe de plantes à partir de ses représentants contemporains peut se faire théoriquement par le biais d'informations provenant d'un, de deux ou de trois des génomes hébergés par les cellules végétales de façon séparée ou combinée. Plusieurs types de molécules sont exploités pour établir des phylogénies ; nous ne développerons ici que les phylogénies basées sur les acides nucléiques qui sont aujourd'hui les plus usitées.

Durant ces vingt dernières années, le génome chloroplastique est apparu comme la source d'information de prédilection pour déterminer les liens de parenté tissés entre les taxons végétaux au cours de leur évolution, en raison notamment de son abondance dans la cellule, de sa petite taille (typiquement entre 120 et 200 kb), de sa richesse en gènes simple copie, et de son mode d'évolution de type conservateur (revue dans Soltis et Soltis 1998). L'engouement des systématiciens pour ce génome, stimulé par les avancées technologiques, s'est traduit par une explosion du nombre de phylogénies et consécutivement de séquences chloroplastiques publiées. Le gène *rbc*L, composé d'un seul exon codant pour la grande sous-unité de la ribulose-1,5-biphosphate carboxylase (RUBISCO), a été largement utilisé pour reconstruire l'histoire évolutive de groupes de plantes de niveau taxonomique élevé, embranchement, classe, ordre ou famille (Clegg 1993). D'autres régions du génome chloroplastique évoluant à plus grande vitesse, notamment parmi les régions non codantes comme les régions intergéniques et les introns, ont rapidement été identifiées (e.g. Taberlet *et al.* 1991; Gielly et Taberlet 1994; Demesure *et al.* 1995; Dumolin-Lapègue *et al.* 1997b), permettant de répondre aux besoins d'autres chercheurs soucieux d'engager la même démarche avec des groupes taxonomiques de rang inférieur tel le genre ou même l'espèce (Figure 1). Aujourd'hui, le génome chloroplastique est le mieux connu des trois génomes de la cellule végétale et on compte déjà 11 espèces de plantes supérieures pour lesquelles la séquence complète est disponible, parmi lesquelles une gymnosperme et 10 angiospermes (trois monocotylédones et sept dicotylédones). Le génome chloroplastique des plantes a eu longtemps la réputation d'être inadapté à l'étude des populations, reposant sur l'hypothèse, tacitement admise mais aujourd'hui réfutée, que le polymorphisme chloroplastique était rare à l'intérieur de l'espèce (Harris et Ingram 1991). Force est de constater aujourd'hui que les variations observées à l'échelle du génome chloroplastique sont largement exploitées pour des études de phylogéographie (e.g. Dumolin-Lapègue *et al.* 1997a; Comes et Abbott 2001) et de phylogénie fine, telles celles ayant servi à montrer l'origine multiple des polyploïdes (e.g. Soltis *et al.* 1989; Doyle *et al.* 1990).

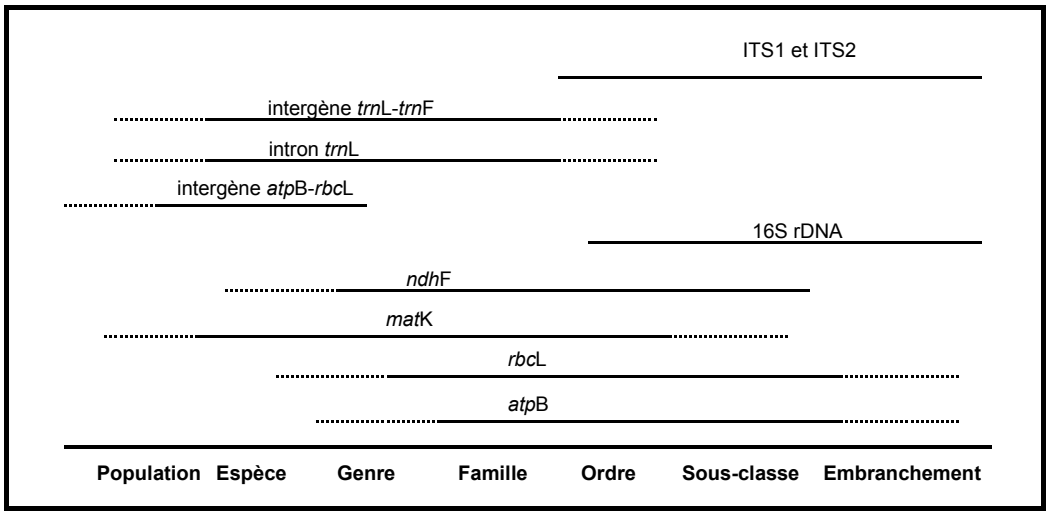

Figure 1 : Choix des régions et gènes chloroplastiques cibles en fonction du niveau taxonomique étudié (d'après Soltis et Soltis 1998).

Le génome mitochondrial des plantes a connu un accueil bien différent de celui du génome chloroplastique de la part des systématiciens. Au sein du règne végétal, la structure du génome mitochondrial (taille, configuration, ordre des gènes) est très variable, ce qui rend son étude plus difficile. Seuls deux génomes mitochondriaux de plantes supérieures ont été séquencés à ce jour. De plus, la vitesse d'évolution des séquences codantes était réputée faible (Palmer 1992) avec un taux de substitution 3 à 8 fois moindre que celui du génome chloroplastique (Tableau 1) (Wolfe *et al.* 1987). Son usage avait donc été limité aux seules études s'adressant à des niveaux taxonomiques hiérarchiquement très élevés (Hiesel *et al.* 1994; Bowe *et al.* 2000; Chaw *et al.* 2000). A l'opposé des idées reçues, des travaux récents ont montré que le taux de substitution variait grandement d'un gène mitochondrial à un autre (Laroche *et al.* 1997), et d'un groupe de plantes à un autre (Palmer *et al.* 2000). Jusqu'alors quelque peu délaissées par les phylogénéticiens, les insertions/délétions, fréquentes dans le génome mitochondrial, se sont parfois révélées phylogénétiquement informatives. Dans le même temps, on a vu apparaître des phylogénies de plantes basées sur des séquences mitochondriales aux niveaux de familles, Orchidaceae (Freudenstein *et al.* 2000; Freudenstein et Chase 2001), ou de genres, *Pelargonium* (Bakker *et al.* 2000) et *Cucurbita* (Sanjur *et al.* 2002). Les polymorphismes de longueur des fragments de restriction du génome mitochondrial sont aujourd'hui fréquemment employés pour distinguer des populations d'une même espèce, chez les gymnospermes (e. g. Wu *et al.* 1998; Sinclair *et al.* 1999) comme chez les angiospermes (e. g. Hong *et al.* 1995; Ronfort *et al.* 1995; Dumolin-Lapègue *et al.* 1998).

Tableau 1 : Comparaison de la vitesse d'évolution des gènes en fonction de leur localisation (d'après Wolfe *et al.* 1987).

Génome	Gène	Espèce	Nombre de substitutions par site (x100)	
		Monocot/Dicot	synonymes	non synonymes
Chloroplastique	*atp*A	BM/EPT	59	8
	*atp*B	BMOR/EPT	66	5
	*atp*E	BMOR/EPT	59	18
	*atp*F	B/EPT	44	13
	*rbc*L	MR/ELPéPT	72	5
	*psa*A	M/EPT	55	2
	*psa*B	M/EPT	50	2
	*psb*B	M/ET	62	2
	*psb*C	M/EPT	53	2
	*psb*D	M/EPT	52	1
	*psb*G	M/ET	60	10
	*pet*A	BR/EOePTV	75	6
	*pet*B	M/ET	64	1
Mitochondrial	*cox*I	MS/OeSj	21	3
	*cox*II	BMR/OePSj	22	7
	cob	BM/Oe	9	3
	*atp*9	M/PéT	28	2
	*atp*A	M/Oe	27	4
	*rpS*13	M/T	19	5
Nucléaire	*gap*C	O/Mo	119	9
		O/T	110	10
	adh	M_1/P	191	11
		M_2/P	>250	12
		M_1/A	202	13
		M_2/A	245	14
	Phytochrome	Oe/C	>250	24

A : arabette, B : blé, C : courgette, E : épinard, L : luzerne, M : maïs (M_1 et M_2 représentent les deux loci *adh* du maïs), Mo : moutarde, O : orge, Oe: *Oenothera*, P : pois, Pé : pétunia, R : riz, S : sorgho, Sj : soja, T : tabac, V : *Vicia faba*,

Le génome nucléaire a été comparativement sous-exploité dans les études de taxonomie. La fréquente duplication des gènes nucléaires et son corollaire, l'existence de gènes *orthologues* (relation entre gènes dérivant d'événements de spéciation seulement) et de gènes *paralogues* (relation entre gènes ayant connu au moins un événement de duplication), sont en partie responsables de cette désaffection. En effet, la reconstruction phylogénétique ne peut être fidèle que si les informations proviennent de loci qui entretiennent une relation d'orthologie. Mais l'orthologie reste souvent difficile à établir. Une démonstration rigoureuse de l'orthologie imposerait, outre une similitude de séquence, la convergence de plusieurs autres indices : identité de fonction, partage d'une même histoire phylogénétique, synténie entre espèces (Doyle et Gaut 2000). Orthologues et paralogues forment ce qu'on appelle une famille de gènes. Parmi les nombreuses familles multigéniques, seuls les gènes nucléaires qui codent pour les ARN ribosomiques (ARNr) ont depuis longtemps intéressé les systématiciens. Présents sous formes d'unités répétées en tandem sur un ou plusieurs loci chromosomiques, ils sont réputés soumis à une *homogénéisation intra- et interloci* (*évolution*

concertée). Chaque unité, dont le nombre peut atteindre plusieurs milliers, est composée de régions soumises à des contraintes évolutives variables qui, à ce titre, peuvent servir de cibles à des systématiciens travaillant à des niveaux taxonomiques différents (Soltis et Soltis 1998). Contrairement aux ADN ribosomiques, les gènes présents en faible nombre de copies dans le génome nucléaire semblent peu soumis à un mode d'évolution concertée (Cronn *et al.* 1999). Plusieurs gènes nucléaires ont déjà été utilisés et semblent intéressants en phylogénie, notamment *adh* (alcool déshydrogénase Small *et al.* 1998; Sang et Zhang 1999; Ferguson et Sang 2001; Cronn *et al.* 2002) et H3-D (histone Doyle *et al.* 1999; Doyle et Doyle 2000; Brown *et al.* 2002). Soumis à une évolution plus rapide que les gènes chloroplastiques et mitochondriaux (Tableau 1), ils semblent particulièrement indiqués pour des phylogénies de genres ou d'espèces lorsque les gènes chloroplastiques sont peu résolutifs (e.g. Small *et al.* 1998; Huang *et al.* 2002b).

Les sources d'incongruence

Le but final des études de phylogénie est de témoigner des relations passées entre toutes les entités d'un groupe, ce qui se traduit souvent graphiquement sous la forme d'une arborescence. Cette représentation permet de rendre compte de deux dimensions, le temps verticalement et la diversité horizontalement. Les entités situées aux extrémités de ces arbres sont les séquences, gènes ou génomes et non l'organisme, la population ou l'espèce dont ils sont issus, bien que cette évidence soit rarement explicitée et bien souvent ignorée en systématique moléculaire (Doyle 1993). Or, inférer les relations historiques entre les espèces à partir des informations fournies par les gènes peut entraîner des erreurs. Diverses causes peuvent être à l'origine d'"*incongruences*" ou de "*discordances*" (quand deux topologies ne s'accordent pas en tous points) entre la phylogénie des gènes et celle des espèces. Trois sources majeures d'incongruence ont pu être identifiées (Tableau 2) (Rieseberg et Soltis 1991; Wendel et Doyle 1998). L'une d'elle, l'hybridation/introgression, affecte tout particulièrement le règne végétal, les plantes étant susceptibles de s'hybrider assez facilement avec des partenaires recrutés en dehors des limites de l'espèce taxonomique (Grant 1981; Gornall 1997). De nombreux et illustres exemples sont là pour témoigner de la fréquence des événements d'hybridation ou d'introgression dans le règne végétal (revue dans Rieseberg et Soltis 1991; Wendel 2000). En présence d'événements d'hybridation/introgression, non seulement les hypothèses à la base des méthodes classiques de reconstruction phylogénétiques ne sont plus respectées mais la forme arborescente devient inadaptée (une forme en *réseau* devenant plus appropriée).

"If a phylogenetic tree is the extension of the normal pattern of animal speciation, plant speciation has often led to the formation of a phylogenetic web." (Grant 1981)

Tableau 2 : Phénomènes conduisant à une incongruence entre la phylogénie vraie de l'organisme et celle inférée à partir d'un jeu de données (d'après Harris et Ingram 1991; Wendel et Doyle 1998).

Origine du phénomène	Causes
Technique	Données insuffisantes
	Choix de la cible (gène)
	Erreur de clonage ou de séquençage
	Echantillonnage des taxa
Evolutive, à l'échelle de l'organisme	Evolution morphologique convergente ou rapide
	Diversification rapide
	Hybridation/introgression
	Lineage sorting
	Transfert horizontal
Evolutive, à l'échelle du gène ou du génome	Recombinaison intragénique
	Confusion orthologie/paralogie
	Interactions interloci et évolution concertée
	Hétérogénéité des vitesses d'évolution entre taxa
	Hétérogénéité des vitesses d'évolution entre sites
	Hétérogénéité du mode d'hérédité entre taxa
	Composition biaisée en bases
	RNA editing
	Non indépendance des sites

Le genre *Actinidia*

Le genre *Actinidia* (Actinidiaceae) doit son nom à un caractère floral, la disposition en rayons des styles. Il est composé d'espèces pérennes, à feuilles caduques, grimpantes ou rampantes, dioïques. Il est endémique d'Asie, le sud-ouest de la Chine représentant son aire principale de diversité (Liang 1983). Certaines espèces, telle *A. polygama*, *A. arguta* et *A. chinensis* sont largement distribuées, leur aire de répartition s'étendant du Japon jusqu'à l'Ouest de la Chine en passant par le Nord-Est de l'Asie (Li 1952). La plupart sont réparties sur un territoire plus restreint (Li 1952). Les premiers spécimens d'*Actinidia* ont été collectés au Népal en 1821 mais ils ne furent baptisés avec leur nom définitif qu'en 1836 (Dunn 1911). Une première description du genre fut faite par Finet et Gagnepain (1905) et une première subdivision en sections a été établie en 1911 par Dunn. Par la suite, trois révisions successives furent proposées, la plus récente datant de 2002 (Annexe 1). Une trentaine de nouvelles espèces ont été découvertes à la suite des prospections réalisées en Chine au cours du XX^{e} siècle et se sont rajoutées aux 24 espèces décrites par Dunn. De plus, certains taxons initialement considérés comme des variétés ont été élevés au rang d'espèces (Liang et Ferguson 1986). Totalisant actuellement 57 espèces, le genre *Actinidia* est divisé en quatre sections (Annexe 2), i. e. *Leiocarpae*, *Maculatae*, *Stellatae* et *Strigosae*, sur la base de caractères du fruit (présence ou absence de lenticelles), de la moelle (lamellée ou non lamellée) et des poils (simples ou en étoile). Toutes les études cytogénétiques réalisées à ce

jour mentionnent un nombre chromosomique de base de x = 29 (Zhang et Beuzenberg 1983; McNeilage et Considine 1989). Le genre *Actinidia* est considéré comme une série polyploïde (Ferguson 1990b), avec des taxons diploïdes, tétraploïdes, hexaploïdes et octoploïdes (Guijun *et al.* 1994; Yan *et al.* 1997c). Des variations infraspéciques du niveau de ploïdie sont déjà connues pour trois espèces : *A. chinensis* (diploïdes et tétraploïdes), *A. arguta* (tétraploïdes, hexaploïdes et octoploïdes) et *A. melanandra* (diploïdes et tétraploïdes) (Yan *et al.* 1997c). Le rôle joué par l'hybridation dans ces doublements chromosomiques n'est pas complètement éclairci et fait l'objet de controverses (Crowhurst *et al.* 1990; Crowhurst et Gardner 1991; Testolin et Ferguson 1997).

Le déterminisme du sexe

Les espèces d'*Actinidia* sont fonctionnellement dioïques : les fleurs femelles sont pourvues d'étamines mais libèrent du pollen non fertile et les fleurs mâles présentent un gynécée réduit généralement démuni de styles, stigmates et ovules (McNeilage 1991a). Pendant la période juvénile, rien ne distingue phénotypiquement un mâle d'une femelle. La maturité sexuelle n'est atteinte qu'après trois à cinq années. Au sein d'une descendance, on observe une ségrégation d'une femelle pour un mâle. Le déterminisme du sexe semble donc monofactoriel, dépendant d'un seul gène ou d'une paire de chromosomes sexuels que rien ne distinguerait cytogénétiquement des autosomes (Testolin *et al.* 1995). La dioécie, bien que généralisée à l'ensemble du genre, est faiblement labile (McNeilage 1991b; McNeilage 1991a; Testolin *et al.* 1995). Le sexe hétérogamétique est le sexe mâle (Testolin *et al.* 1995).

La domestication

A. chinensis et son apparentée *A. deliciosa*, toutes deux membres de la section *Stellatae*, sont actuellement les seules espèces qui ont un intérêt économique significatif. La baie d'*A. chinensis* appelée "mihoutao" en Chine fait encore l'objet de cueillette dans les régions où l'espèce est présente à l'état sauvage. La baie d'*A. deliciosa*, espèce introduite en Nouvelle Zélande au début du XX^{e} siècle et pour cette raison dénommée kiwifruit (le kiwi est le nom commun d'un oiseau endémique emblème du pays), est produite en vergers commerciaux, vendue et désormais connue dans le monde entier (Ferguson et Bollard 1990). *A. deliciosa* ainsi que *A. setosa* ont été longtemps considérées avec comme de simples variétés d'*A. chinensis*, elles sont traitées comme deux espèces distinctes dans les récentes classifications. *A. setosa*, espèce insulaire confinée à Taiwan, est isolée des deux autres qui sont continentales, *A. chinensis* localisée dans la partie Est et *A. deliciosa* dans la partie Ouest de la Chine (Ferguson 1990a). En outre, elles sont morphologiquement et cytogénétiquement distinctes (Liang et Ferguson 1986; Huang et Ferguson 2001). *A. chinensis* comprend des cytotypes di- et tétraploïdes (Guijun *et al.* 1994) tandis qu'un seul cytotype est à ce jour rapporté pour *A. setosa* et *A. deliciosa*, respectivement di- et

hexaploïde (Yan *et al.* 1997c). Pilosité et morphologie du bourgeon hivernant sont des critères spécifiques (Ferguson 1990a). En dépit de ces caractères distinctifs, tous les taxonomistes ne sont pas d'accord pour attribuer le statut d'espèce à ces trois taxons. Par contre, certains considèrent *A. chinensis* et *A. deliciosa* comme un complexe d'espèces (Testolin *et al.* 1997).

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Strict paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in intraspecific crosses of kiwifruit

J. Chat, L. Chalak, R. J. Petit 1999
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Résumé

Des études ont établi que les chloroplastes sont hérités paternellement dans des croisements interspécifiques au sein d'*Actinidia*. Cependant, des problèmes de fécondation inhérents aux croisements interspécifiques sont susceptibles de perturber la transmission des organites. Six clones femelles, i. e. 'Abbott', 'Bruno', 'Greensill', 'Hayward', 'Jones', 'Monty', et quatre clones mâles ont été utilisés pour identifier des polymorphismes de l'ADNcp au sein de l'espèce cultivée de kiwi *A. deliciosa*. Le profil d'un fragment chloroplastique amplifié par PCR à l'aide d'amorces universelles et digéré par *Hpa*II a révélé un polymorphisme au niveau intraspécifique. L'hérédité de l'ADN chloroplastique a été étudiée dans 143 descendants issus de trois croisements intraspécifiques. Tous les descendants présentaient le profil de restriction du parent mâle, indiquant que l'hérédité maternelle de l'ADNcp, si elle existe, est rare chez le kiwi. Une hérédité maternelle stricte de l'ADNmt a été confirmée à l'aide des mêmes croisements que ceux utilisés pour l'ADNcp. Les études d'hérédité cytoplasmique au sein du genre *Actinidia* représentent à ce jour le cas le mieux documenté d'hérédité contrastée des génomes cytoplasmiques chez les angiospermes.

Préambule

L'observation des descendances de plantes présentant des décolorations (déficiences chlorophylliennes) chez le *Pelargonium* fut à l'origine de la découverte par Baur en 1909 du mode d'hérédité non mendélien des organites cytoplasmiques que sont les chloroplastes (Hagemann 2000). La même année furent démontrées l'hérédité biparentale chez *Pelargonium* (Baur 1909) et l'hérédité maternelle chez *Mirabilis*, *Urtica*, et *Lunaria* (Correns 1909). Un nombre croissant de groupes de plantes a été étudié depuis à l'aide de marqueurs phénotypiques (panachure, stérilité, résistance à des composés chimiques). Plus récemment, l'utilisation de marqueurs génétiques (polymorphisme de longueur des fragments de restriction, microsatellites) a permis d'établir le mode de transmission dans des groupes de plantes pour lesquels aucun marqueur phénotypique chloroplastique n'était connu.

La tendance générale est une hérédité des organites (ADNmt et ADNcp) maternelle chez les angiospermes et paternelle chez les gymnospermes (revue par Corriveau et Coleman 1988; Harris et Ingram 1991). Cependant, de nombreuses exceptions existent. Chez les Pinaceae, le chloroplaste est hérité du père et la mitochondrie de la mère (Wagner *et al.* 1987; Neale et Sederoff 1989; Wagner *et al.* 1991). Pour certaines angiospermes, i. e. *Medicago* (Masoud *et al.* 1990), *Pelargonium* (Tilney-Bassett et Birky 1981), *Petunia* (Derepas et Dulieu 1992) et *Nicotiana* (Medgyesy *et al.* 1986), le chloroplaste présente une hérédité biparentale avec un biais maternel plus ou moins marqué selon les espèces. Dans de très rares cas, i.e. *Musa* (Fauré *et al.* 1994) et *Cucumis* (Havey *et al.* 1998), on observe une hérédité paternelle du génome mitochondrial.

L'hypothèse selon laquelle le mode de transmission des chloroplastes est uniforme est implicite dans bon nombre d'études de systématique moléculaire, que le groupe étudié soit une espèce, un genre, une famille ou même un taxon de niveau hiérarchique supérieur. Le non-respect de cette hypothèse peut pourtant avoir des conséquences dramatiques sur la reconstruction phylogénétique, particulièrement lorsque l'on s'intéresse aux scénarios d'hybridation/introgression et de polyploïdisation (Harris et Ingram 1991). Or, le mode d'hérédité des organites a changé fréquemment au cours de l'évolution (Sears 1980; Birky 1995). Il pourrait également influer sur la vitesse d'évolution du génome cytoplasmique, le taux de substitution semblant plus grand lorsque l'organite est hérité paternellement (Whittle et Johnston 2002).

L'étude du mode d'hérédité des organites au sein du genre *Actinidia* constituait donc un préalable à l'établissement d'une phylogénie moléculaire basée sur ces génomes. Dans le genre *Actinidia*, les études réalisées sur des hybrides interspécifiques

montraient que l'ADN chloroplastique était hérité strictement du père (Figure 2) et l'ADN mitochondrial strictement de la mère (Cipriani *et al.* 1995; Testolin et Cipriani 1997). Le kiwi constituait donc un cas unique parmi les angiospermes. Nous nous sommes attachés ici à confirmer ce mode d'hérédité non conventionnel sur des hybrides intraspécifiques.

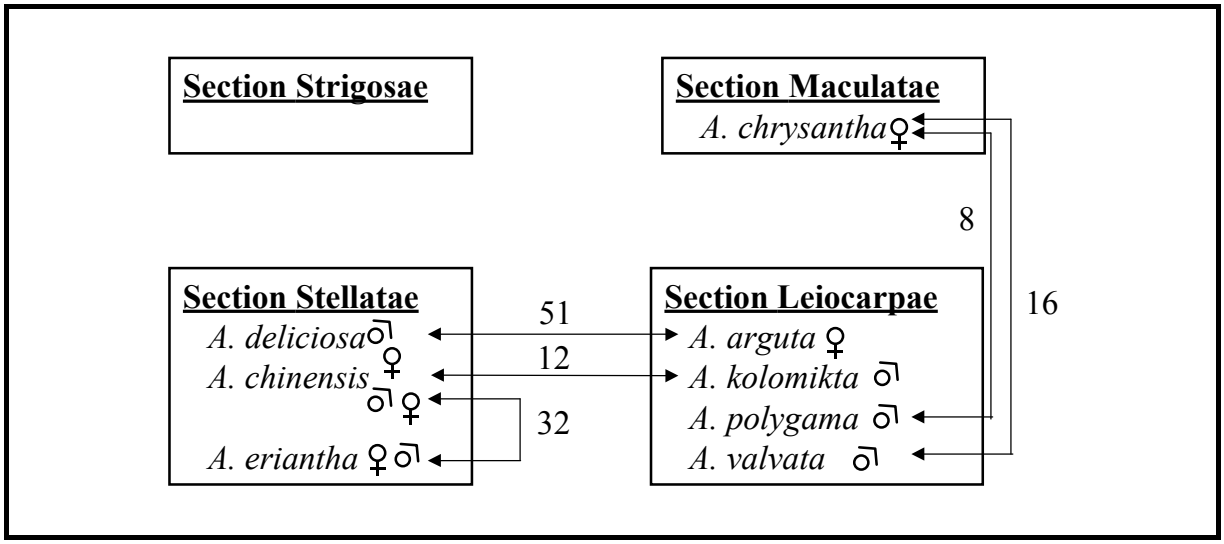

Figure 2 : Croisements interspécifiques pour lesquels une hérédité chloroplastique paternelle stricte a été démontrée au sein du genre *Actinidia*. Sont indiquées les noms des espèces utilisées dans les croisements et l'effectif des descendances analysées (d'après Cipriani *et al.* 1995; Testolin et Cipriani 1997).

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Strict paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in intraspecific crosses of kiwifruit

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Abstract Previous studies have established that chloroplasts are inherited paternally in *Actinidia* interspecific crosses. However, fertilisation problems in interspecific crosses may affect the transmission of organelles. Six female clones, i.e. 'Abbott', 'Bruno', 'Greensill', 'Hayward', 'Jones', 'Monty', and four male clones were used to identify cpDNA polymorphisms within the cultivated kiwifruit species *A. deliciosa*. The restriction patterns by *Hpa*II of a chloroplast fragment amplified by PCR with a pair of universal primers revealed a polymorphism at the intraspecific level. The inheritance of cpDNA in 143 seedlings from three intraspecific crosses in kiwifruit (*Actinidia deliciosa*) was studied. All offspring displayed the restriction pattern of the paternal parent, indicating that maternal inheritance of cpDNA in kiwifruit is rare at best. Strict maternal inheritance of mtDNA was confirmed in the same crosses used to investigate cpDNA transmission. Studies of cytoplasmic inheritance in the *Actinidia* genus represent to date the best documented report of differential organelle inheritance of cpDNA and mtDNA in angiosperms.

Key words *Actinidia deliciosa* · cpDNA · mtDNA · PCR · Universal primers

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Introduction

The *Actinidia* genus, native to China (Li 1952), consists of more than 50 species and forms a polyploid series with the basic chromosome number of 29 (Jie and Beuzenberg 1983; Yan et al. 1997). All members of the *Actinidia* genus are perennial climbing or straggling plants and all appear to be functionally dioecious (McNeilage 1991). Pollen is transferred from male to female flowers both by wind and by insects (Costa et al. 1993). Botanical literature is very confusing on a number of points, and the systematic position of the genus *Actinidia* has not been completely resolved until now. The *Actinidia* genus seems to be closely related to the genera *Clematoclethra* and *Saurauia*, with all three generally considered as constituting the family Actinidiaceae, itself included in the order Theales (Dunn 1911). The other conflicting point concerns the name of the cultivated species. Kiwifruit was originally classified as a variety of *Actinidia chinensis*, i.e. var 'hispida', but differences in morphological traits as well as in chromosome number have recently led Liang and Ferguson (1984) to consider the existence of two distinct species: *A. chinensis* (mainly $2n = 2x = 58$) and *A. deliciosa* ($2n = 6x = 174$). At present, they are the only two *Actinidia* species of economic importance.

The kiwifruit industry is a recent culture based on a single female cultivar of *Actinidia deliciosa*, the 'Hayward' variety. This cultivar was selected in New Zealand in 1930 following one or two generations of breeding following a first introduction of seeds from China in 1904 (Ferguson and Bollard 1990). 'Hayward' is the only cultivar grown to a significant extent both in New Zealand and throughout the world. The reason for this predominance is that 'Hayward' produces fruits of a good size, shape and flavour that can be stored for long periods of time. Nevertheless, breeding programmes have been undertaken to create new cultivars in New Zealand, and to a lesser extent in other producing countries.

In China, fruits of *A. chinensis* were first simply collected from the wild, to be sold locally or for home consumption (Ferguson 1990). Recent research and experimental efforts in China have been directed towards selecting high-quality plants within *A. chinensis* (Ch'ang 1982). An attempt to preserve part of the existing natural resources has also been undertaken at local and national levels (Huang et al. 1997).

An exact knowledge of the genetic diversity of the genus *Actinidia* is required, not only for the development of efficient conservation strategies but also for the design of breeding programmes. Molecular analyses of cytoplasmic DNA are useful tools for evaluating diversity and phylogeny among *Actinidia* species. The determination of chloroplast and mitochondria inheritance is a pre-requisite for the use of organelle DNA molecules in tracing the evolutionary history of plant species. In the past, precise information on organelle genome inheritance in plants has been limited to a few species due to the lack of phenotypic markers. More recently, the development of molecular markers has been of great utility for investigating organelle genome polymorphism and inheritance (Reboud and Zeyl 1994), particularly in species with unknown plastid mutants. With a plastid mutation such as chlorophyll deficiency, a large number of plants can be observed, thereby increasing the chance to detect rare transmission events (Ohba et al. 1971; Hagemann 1992). On the other hand, the use of such phenotypic markers can induce a bias in favour of the wild-type allele, as has been established in *Pelargonium* by Tilney-Bassett and Birky (1981).

Analyses of cytoplasmic transmission within the *Actinidia* genus have been recently assessed. Surprisingly, the independent inheritance of chloroplast and mitochondria has been observed within the genus *Actinidia*: chloroplast and mitochondria appear to be paternally and maternally inherited, respectively (Cipriani et al. 1995; Testolin and Cipriani 1997). In gymnosperms, following the early discovery of Ohba et al. (1971) of the predominantly paternal inheritance of chloroplasts in *Cryptomeria japonica*, there have been many other reports of the paternal inheritance of chloroplasts. But to our knowledge, *Actinidia* is the only genus of angiosperms where a purely paternal mode of plastid inheritance has been reported. Considering the importance of the finding of Testolin and Cipriani with respect to plastid genetics in angiosperms, it seemed desirable to confirm this unusual result in other *Actinidia* crosses and to increase the overall sample size.

Cipriani et al. (1995) investigated the mode of inheritance of mitochondria in the genus *Actinidia* in both interspecific and intraspecific controlled crosses. On the other hand, Testolin and Cipriani (1997) determined the mode of inheritance of chloroplasts only for interspecific hybrids, as it seemed difficult to detect intraspecific polymorphism among the *A. deliciosa* clones. The study presented here was undertaken to confirm the paternal inheritance of chloroplast DNA (cpDNA) within the *A. deliciosa* cultivated species. We therefore analysed four intraspecific crosses of *A. deliciosa* involving a total of 150 offspring using polymerase chain reaction (PCR) amplification of non-coding chloroplast regions and subsequent restriction fragment analysis. The same crosses were used to confirm the maternal inheritance of mitochondrial DNA (mtDNA).

Materials and methods

Parental plants

The present study involved several female and male clones, all belonging to the species *A. deliciosa*. Six female clones selected in New Zealand and introduced into France (INRA Bordeaux) from New Zealand in 1970 were evaluated, i.e. 'Abbott', 'Bruno', 'Greensill', 'Hayward', 'Jones' and 'Monty' (for precise descriptions, see Jie and Thorp 1986). Four male clones, expected to be different from each other, are referred to as M1, M2, M3 and M4 in the text. M1 is the Italian rootstock selection 'D uno', and M2 is a New Zealander pollinator selection named 'Tomuri' (Chalak and Legave 1997). Two other unnamed male clones, M3 and M4, were used.

Sexual crosses

Dioecy is a general although not absolute feature in the genus *Actinidia* (Ferguson 1990). Female plants have pistillate flowers with sterile pollen grains, and male plants have staminate flowers with rudimentary pistils (Polito and Grant 1984). The only cases of hermaphrodism reported until now in *A. deliciosa* species have been the isolated occurrence of male plants used as pollinators in the orchards and bearing small fruits (McNeilage 1991). Hermaphrodite flowers have never been reported in *A. deliciosa* female cultivars. Thus self-fertilisation is prevented, and there is no need of emasculation in crossing experiments. The controlled crosses were performed at the INRA research station of Bordeaux and San Giuliano (France) in 1990 and 1992, respectively. Before flowering, both male and female flowers were bagged to exclude pollen transport by honeybees or wind. At male anther dehiscence, pollination was achieved by brushing the stigma of the female flowers with the anthers of the male flowers.

DNA extraction

Young leaves or buds were harvested in the spring and immediately frozen. Total genomic DNA was extracted and purified according to a modified CTAB procedure (Saghai-Maroof et al. 1984). Frozen tissue was ground into powder with a mechanical mill, incubated in tubes containing 9 ml of extraction buffer (100 m*M* TRIS pH 8.0, 1.4 *M* NaCl, 20 m*M* EDTA, 2% hexadecyltrimethylammonium bromide, 0.1% 2-mercaptoethanol) and agitated gently at 60°C for 60 min. The tubes were cooled before adding 4 ml of chloroform/octanol, 24 : 1 (v/v). After centrifugation at 5000 *g* for 10 min, the upper aqueous phase was treated with 2.5 U of ribonucleases A for 30 min. The final DNA stock solution was stored at − 20°C and diluted 10–100 times before use.

Restriction fragment length polymorphism (RFLP) of organelle genome

The template for PCR amplification consisted of 4 ng of genomic DNA. The reaction buffer (25 µl) contained 75 m*M* TRIS-HCl,

Table 1 Description of the primers and restriction enzymes used in this study

Primer 1	Primer 2	References	Abbreviation
Chloroplast primers			
*psb*C [psII 44-kDa protein]	*trn*S [tRNA-Ser (UGA)]	Demesure et al. 1995	CS
*trn*D [tRNA-Asp (GUC)]	*trn*T [tRNA-Thr (GGU)]	Demesure et al. 1995	DT
*trn*T [tRNA-Thr (GGU)]	*psb*C [psII 44-kDa protein]	Dumolin-Lapègue et al. 1997	TC
Mitochondrial primers			
*nad*1 exon B	*nad*1 exon C	Demesure et al. 1995	*nad*1-B/C

1.8 m*M* $MgCl_2$, 5 µg of BSA, 20 m*M* $(NH_4)_2SO_4$, 0.01% (w/v) Tween 20, 0.1 m*M* of each of the four dNTP, 0.2 µ*M* of each primer and 0.4 U of *Taq* polymerase (Goldstar, Eurogentec). A description of the primers is given in Table 1. These pairs of primers have been chosen because they successfully amplified cytoplasmic DNA of a wide range of plant species belonging to angiosperms and gymnosperms (Demesure et al. 1995). The amplification was carried out in a 96-well Perkin Elmer GeneAmp PCR System 9600 (details of the amplification conditions are given in Demesure et al. 1995 and Dumolin-Lapègue et al. 1997). The amplified fragments were digested by a single restriction enzyme (*Hpa*II or *Hin*fI) according to the manufacturer's recommendation. The restriction fragments were separated by electrophoresis on 8% polyacrylamide gels in a 1 × TBE buffer, stained in ethidium bromide and visualised by UV light. The 1-kb ladder of GibcoBRL (Life Technologies) was used as the molecular-weight marker.

Random amplified polymorphic DNA (RAPD)

The arbitrary sequence 10-mer primers used were obtained from Operon Technologies (Almeda, Calif.). The primer finally chosen for parentage analysis was OPQ-09 (5′-GGCTAACCGA-3′). The PCR reaction mixture (18 µl) contained 20 m*M* TRIS-HCl (pH 8.4), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 3.6 µg of BSA, 100 µ*M* each of dATP, dTTP dCTP, and dGTP, 0.7 U of *Taq* DNA Polymerase (Life Technologies™), 1 ng of primer and 30 ng of template DNA. PCR was performed in the same DNA thermocycler as above using 1 cycle of 5 min at 95°C; 45 cycles of 10 s at 95°C, 15 s at 37°C, 2 min at 72°C; and 1 cycle of 4 min at 72°C. The fragments generated by amplification were separated according to size on 1.5% agarose gels in a 1 × TAE buffer, stained and visualised in the same way as the polyacrylamide gels.

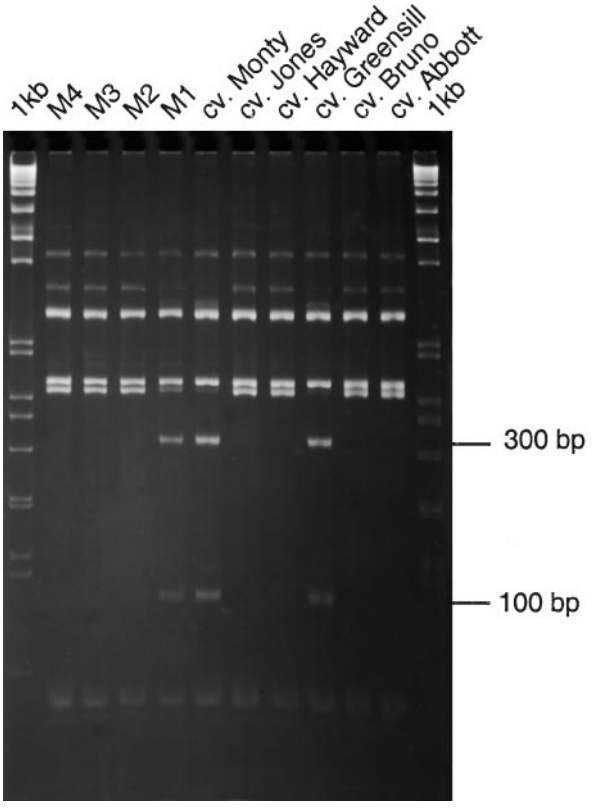

Fig. 1 Chloroplast DNA polymorphism detected at the intraspecific level with CS primers and the *Hpa*II restriction enzyme

Results

Organelle polymorphism within *A. deliciosa*

Polymorphic patterns for mtDNA were revealed among *A. deliciosa* clones using the *nad*1-B/C-*Hin*fI combination (data not shown). This polymorphism appears to be due to an insertion/deletion event estimated to be 30 bp in size. The male clone M1 and all the female cultivars could be distinguished from the remaining three male clones. Polymorphism for cpDNA was detected among *A. deliciosa* clones using the CS-*Hpa*II combination. 'Abbott', 'Bruno', 'Hayward', 'Jones', M2, M3 and M4 showed the same haplotype, which differed from that of 'Greensill', 'Monty' and M1 (Fig. 1). This polymorphism seems to be due to an extra *Hpa*II recognition site.

These intraspecific restriction fragment length polymorphisms provided markers for distinguishing the parental cpDNA and mtDNA haplotypes and were used to demonstrate the mode of organelle inheritance. Among all the potentially informative crosses within *A. deliciosa*, only four crosses were available at the INRA Institute, i.e. 'Greensill' × M3, 'Greensill' × M4, 'Hayward' × M1 and 'Hayward' × M2. Interestingly, two crosses among the four were informative with respect to both mitochondria and chloroplast inheritance (Tables 2 and 3).

Table 2 Restriction pattern of mtDNA revealed by *nad*1-B/C primers and the *Hin*fI restriction enzyme among the six parents of the controlled crosses and subsequently used for determining the mode of mtDNA inheritance

Cross	Parents				Progeny		
	Amplified fragment (in bp)		Polymorphic restriction fragments (in bp)		Restriction pattern		
	Female	Male	Female	Male	Maternal	Paternal	Biparental
Greensill × M3	1676	1706	530	560	40	0	0
Greensill × M4	1676	1706	530	560	61	0	0
Hayward × M1	1676	1676	530	530	–	–	–
Hayward × M2	1676	1706	530	560	7	0	0
				Total	108	0	0

Table 3 Restriction pattern of cpDNA revealed by CS primers and the *Hpa*II restriction enzyme among the six parents of the controlled crosses and subsequently used for determining the mode of cpDNA inheritance

Cross	Parents			Progeny		
	Amplified fragment (in bp)	Chloroplastic polymorphic restriction fragments (in bp)		Restriction pattern		
		Female	Male	Maternal	Paternal	Biparental
Greensill × M3	1600	300 + 100	400	0	40	0
Greensill × M4	1600	300 + 100	400	0	61	0
Hayward × M1	1600	400	300 + 100	0	42	0
Hayward × M2	1600	400	400	–	–	–
			Total	0	143	0

Maternal inheritance of mtDNA, in intraspecific crosses of *A. deliciosa*

The informative crosses and the corresponding diagnostic fragments are summarised in Table 2. As seen in Fig. 2, *Hin*fI digests produced a 530-bp band unique to the female clone 'Greensill' and a 560-bp band unique to the male clone M3. That polymorphism allowed the study of mtDNA inheritance in 108 offspring from three crosses. Only the mtDNA patterns of the female clones were observed among the 108 offspring (Fig. 2 and Table 2).

Paternal inheritance of cpDNA in intraspecific crosses of *A. deliciosa*

The polymorphic restriction patterns between the parents are given in Table 3 for the CS-*Hpa*II combination. This polymorphism seems to be due to an extra *Hpa*II recognition site in the female clone 'Greensill' and the male clone M4 that results in a 400-bp fragment being cleaved into a 300-bp and a 100-bp fragment (Fig. 3). As indicated in Fig. 3 and Table 3, all 143 offspring from the three informative crosses exhibited the paternal pattern.

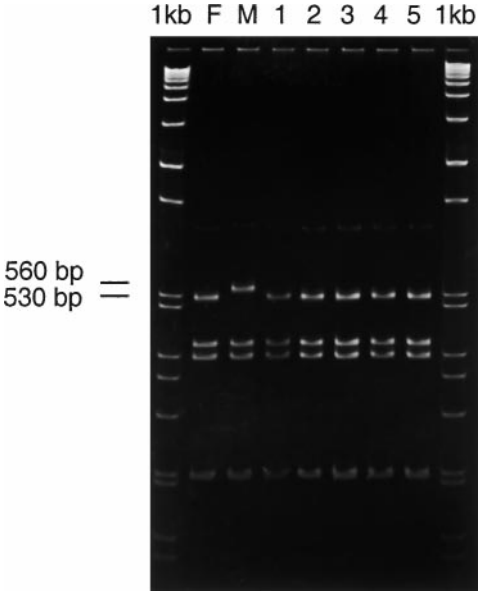

Fig. 2 Maternal inheritance of mtDNA in a progeny from the cross 'Greensill' × M3. The amplified fragment obtained using *nad*1-B/C primers was digested by the *Hin*fI restriction enzyme. The male parent M3 (*M*) differs from the female parent 'Greensill' (*F*) and the progeny (*lanes 1–5*) by a deletion of 30 bp in the first restriction fragment. *1kb* Molecular-weight marker

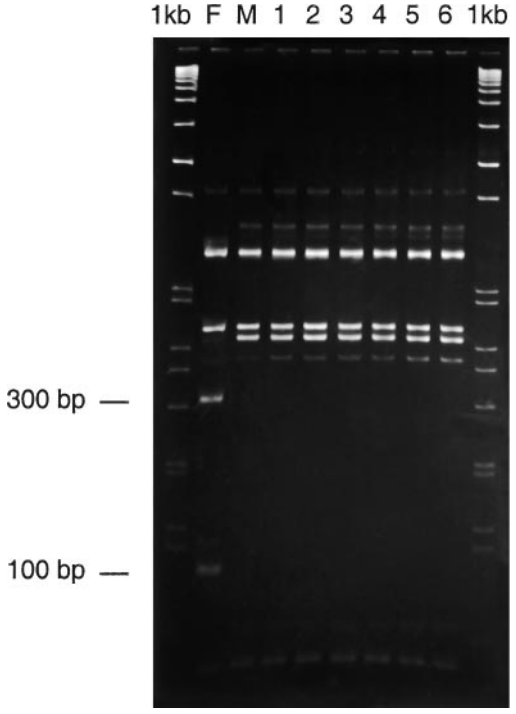

Fig. 3 Paternal inheritance of cpDND in a progeny from the cross 'Greensill' × M4. A 1600-bp cpDNA fragment was amplified by PCR with the CS primers and digested by the *Hpa*II restriction enzyme. The female parent 'Greensill' (*F*) differs from the male parent M4 (*M*) and the progeny (*lanes 1–6*) by two additional bands, 100 bp and 300 bp, expected to result from an extra *Hpa*II restriction site. *1kb* Molecular-weight marker

Occurrence of pollen contamination

Evidence of pollen contamination was detected among the 'Hayward' × M2 non-informative cross using the CS-*Hpa*II combination. Of the 7 offspring analysed, 1 exhibited an unexpected chloroplast pattern of neither paternal nor maternal origin.

RAPD primers were used to amplify total DNA from the three clones used as parents in Corsica, i.e. 'Hayward', M1 and M2, in an attempt to identify which male clone was the parent of the aberrant offspring. Among the 20 primers tested, 1 (OPQ-09) detected clear polymorphisms distinguishing the three parents and was subsequently chosen to conduct a parentage test in both 'Hayward' × M1 and 'Hayward' × M2 progeny. The average length of the amplified bands was calculated to be 1310 bp for the M1-specific major band and 500 bp for the M2-specific major band (Fig. 4). Each RAPD marker segregated in the respective progeny, indicating that the two loci amplified were of nuclear origin. The questionable seedling of the cross 'Hayward' × M2 exhibited the M1-specific major band but not that of M2. On the contrary, all the other offspring patterns were consistent with the respective pedigree. Since the unexpected offspring had also the same cpDNA type as the male M1, contamination by pollen from this clone must have taken place. The occurrence of pollen contamination during artificial pollination may be explained by the fact that the controlled crosses involving the paternal clones M1 and M2 were made the same day by the same person in the same orchard.

Discussion

We studied the inheritance of chloroplast and mitochondrial genomes in *A. deliciosa* using restriction polymorphisms of PCR-amplified cpDNA and mtDNA fragments. The use of PCR to detect intraspecific organelle DNA polymorphisms and to investigate their mode of inheritance in plants is now possible thanks to the availability of 'universal primers' (Demesure et al. 1995; Taberlet et al. 1991). 'Universal primers' have been designed to match conserved sequences, such as tRNA genes or DNA coding regions, and then amplify mostly DNA noncoding regions. Such noncoding regions of organelle DNA are expected to evolve more rapidly than coding regions and thus to reveal more polymorphism. Genetic analysis using PCR amplifications is fast and reliable and requires very small amounts of DNA. The strict uniparental inheritance pattern observed in our study confirms that none of the DNA fragments amplified was of nuclear origin.

Two pairs of primers were used, the first one to investigate the mode of inheritance of cpDNA in *A. deliciosa*, the second one to confirm the previous finding of maternal inheritance of mtDNA previously pointed out in 32 *A. deliciosa* offspring from two crosses (Testolin and Cipriani 1997). For cpDNA, the CS pair of primers selected amplifies a DNA region between the protein gene *ps*II and the tRNA-Ser(UGA) gene (Demesure et al. 1995). The restriction pattern for the CS fragment with the *Hpa*II enzyme revealed an informative polymorphism at the intraspecific level. To our knowledge, this is the first time that intraspecific cpDNA polymorphism has been reported within the *A. deliciosa* species. For mtDNA, the pair of primers selected amplifies an intron of the *nad*1 gene located between exon B and C (Demesure et al. 1995). Testolin and Cipriani (1997) have already reported a mtDNA polymorphism within the amplified DNA fragment *nad*1 at this taxonomic level.

Paternal inheritance of cpDNA in the *Actinidia* genus had been demonstrated previous to this study using interspecific hybrids (Cipriani et al. 1995; Testolin and Cipriani 1997). However, an abnormal nuclear background may affect the inheritance of the organelles, as has been demonstrated in *Festuca* × *Lolium* intergeneric hybrids by Kiang et al. (1994). In the present study, with the exception of 1 offspring probably sired by contaminating pollen, the restriction pattern of

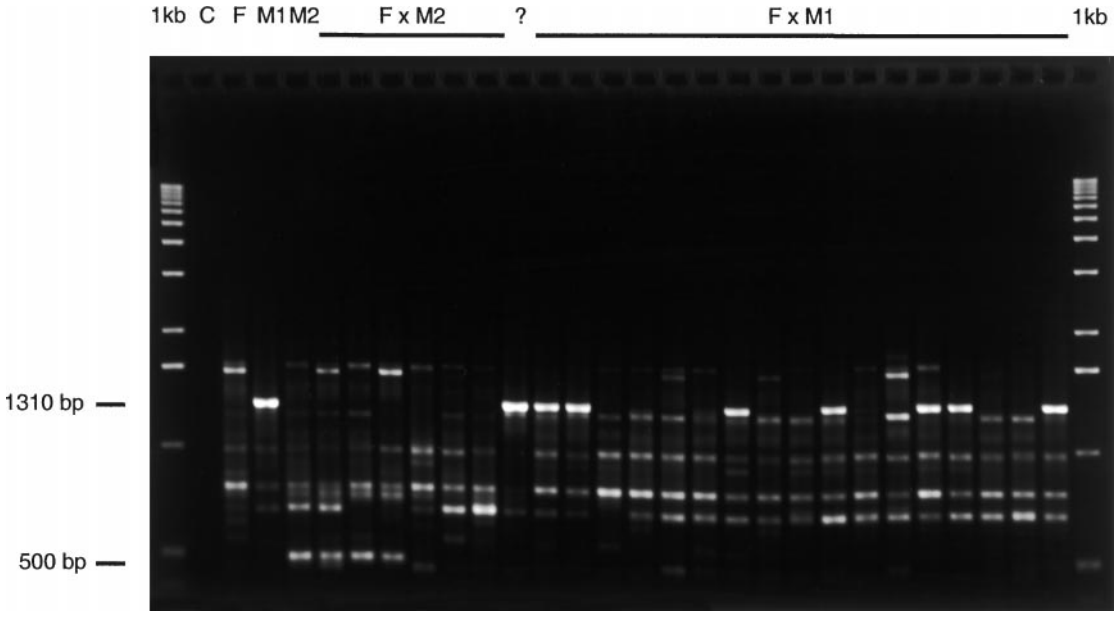

Fig. 4 RAPDs produced with primer OPQ-09 from DNA of the female parent 'Hayward' (*F*), the male parents (*M1* and *M2*), the controlled hybrids ($F \times M1$ and $F \times M2$) and the questionable hybrid (*?*). The PCR product bands referred to in the text are indicated with the approximate base pair lengths. *1kb* Molecular-weight marker, *C* control PCR without template DNA

PCR-amplified cpDNA or mtDNA fragments revealed a unique pattern among all the *A. deliciosa* offspring from a given cross. Our data confirm the predominantly if not strictly maternal transmission of mitochondria previously observed within *A. deliciosa* by Testolin and Cipriani (1997) and provide evidence for a predominantly if not strictly paternal transmission of the chloroplast at the same taxonomic level. In the present study, the mode of inheritance of mtDNA and cpDNA was established from 143 and 108 offspring respectively, each involving three intraspecific crosses in kiwifruit.

Organelle inheritance studies are often documented by the study of progeny derived from controlled crosses. Here, as in many other reports, all progeny appear to inherit a particular organelle type from the same parent. Due to the restricted number of offspring that can be studied, rare cases of leakage from the other parent may easily be overlooked. For instance, a drug resistance marker has been used to show that very rare biparental chloroplast inheritance does occur in *Nicotiana* (Medgyesy et al. 1986), a taxa which was previously considered to have exclusively uniparental-maternal chloroplast inheritance (Maliga et al. 1975; Tilney-Bassett 1978). In conifers, although a largely uniparental-paternal mode of inheritance has been confirmed for cpDNA in many species, occasional offspring with maternal or biparental cpDNA genotypes have been observed, for example in *Pinus banksiana* (Wagner et al. 1989) and *Calocedrus decurrens* (Neale et al. 1991).

Because rare cases of biparental transmission can have disproportionate evolutionary importance (Dumolin-Lapègue et al. 1998), it is important to distinguish between two alternative modes of organelle inheritance: a strict uniparental inheritance, in which the organelle is always derived from the same parent, versus a predominantly uniparental inheritance in which the organelle is occasionally inherited from the other parent. We used the binomial model to evaluate the maximum rate of transmission (P) from the other parent (Milligan 1992) by the following formula:

$$P = 1 - (1 - \beta)^{1/N}$$

where N is the total number of progeny, and β the power of the test.

For this study, we conservatively selected a low probability ($1 - \beta = 0.01$) of falsely accepting the strict uniparental inheritance hypothesis. Considering the absence of maternal cpDNA types among the N = 143 offspring analysed, the maximal rate of maternal transmission of cpDNA that may have been overlooked with these sample sizes is 3.2% (Fig. 5). If we take into account the previous results of Cipriani et al. (1995) and Testolin and Cipriani (1997), based on 56 and 63 interspecific hybrids, respectively, the total number of offspring examined for cpDNA inheritance within the *Actinidia* genus reaches 262, and the corresponding rate drops to 1.7% (Table 4). For levels of 0.5% and 1% of maternal chloroplast leakage, the probabilities of observing at least 1 offspring containing maternally derived chloroplasts in an array of 262 offspring are 73% and 93%, respectively, and the

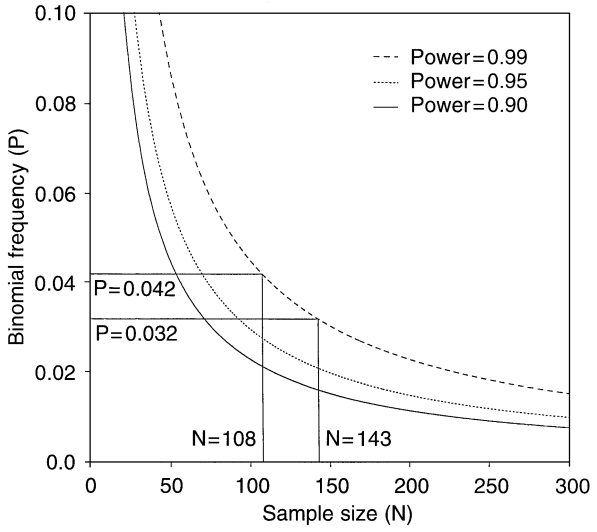

Fig. 5 Relationship between probability of atypical events of organelle transmission (P) and size of the progeny examined (N) for three different powers. This binomial model corresponds to the case where no progeny containing maternally derived chloroplasts or paternally derived mitochondria are found in the progeny sample

true rate of maternal transmission of chloroplasts is therefore probably much lower than 1.7%.

Similarly, the absence of plants characterised by paternal mtDNA types among the N = 108 offspring observed indicates that the true rate of paternal inheritance is lower than 4.2% for the same power of 0.99 (Fig. 5). If we take into account the previous results of Testolin and Cipriani (1997), based on 32 and 102 interspecific and intraspecific hybrids, respectively, the total number of individuals examined within the *Actinidia* genus reaches 242, and the corresponding maximum probability of paternal transmission drops to 1.9% (Table 4).

Altogether, the results provide good evidence for a strong bias towards maternal transmission of mtDNA and paternal transmission of cpDNA within *A. deliciosa*. These results do not agree with the commonly observed uniparental-maternal inheritance of both chloroplast and mitochondrial genomes in angiosperms. Several studies had revealed exceptions to the uniparental-maternal pattern among angiosperms, especially for cpDNA. This genome has been shown to be inherited biparentally, at a high frequency, as in alfalfa (Masoud et al. 1990; Smith 1989) and in *Pelargonium* (Tilney-Bassett and Birky 1981), or at a low frequency, as in *Petunia* (Derepas and Dulieu 1992) and in *Nicotiana* (Medgyesy et al. 1986). But the only case of uniparental-paternal inheritance reported was in the genus *Daucus* (Boblenz et al. 1990), and this was invalidated by Steinborn et al. (1995). With respect to mtDNA, uniparental-paternal inheritance was reported in bananas by Fauré et al. (1994) and in melon by Havey et al. (1998) using 34 and 38 intraspecific hybrids, respectively. This study on kiwifruit represents therefore the most thorough investigation of a case of strict uniparental-paternal inheritance of organelles in angiosperms. The experimental design used in all the chloroplast inheritance studies on kiwifruit involved three intraspecific and eight interspecific crosses and includes 119 intraspecific and 143 interspecific hybrids.

From a cytological point of view, the paternal chloroplast inheritance described in this paper requires the presence of chloroplasts within the pollen grains of *A. deliciosa*. To our knowledge, the only reports on organelle content in pollen grains of the genus *Actinidia* are those of Messina (1993) and Matsunaga et al. (1996). Chloroplasts, as well as mitochondria, are visible in microspore of *A. deliciosa* under transmission electron microscopy (Messina 1993). The cytological observations made by Matsunaga et al. (1996) revealed the presence of large and small fluorescent spots after DAPI staining in the generative cell of the male-derived pollen grain, Consequently, these authors hypothesised

Table 4 Summary of organelle inheritance studies conducted within *Actinidia* genus based upon molecular evidence

Mode of organelle inheritance	Number of crosses (number of progeny) analysed			Total number of progeny	P[a] (%)
	Cipriani et al. (1995)	Testolin and Cipriani (1997)	Present study		
Maternally inherited mitochondria:					
Interspecific crosses	–	4 (102)	–	102	
Intraspecific crosses	–	2 (32)	3 (108)	140	
				242	1.9%
Paternally inherited chloroplast:					
Interspecific crosses	4 (63)	4 (56)	–	119	
Intraspecific crosses	–	–	3 (143)	143	
				262	1.7%

[a] Maximum degree of paternal mitochondrial transmission and maternal chloroplast transmission on the basis of the binomial model curve illustrated in Fig. 5 with a power $\beta = 0.99$

the presence of chloroplasts and mitochondria in the generative cell. The presence of chloroplast nuclei within the generative cell of the male-derived pollen grain of *A. deliciosa* supports the paternal inheritance of the chloroplast previously described. On the other hand, the presence of mitochondria suggest the existence of two separate mechanisms of organelle selection leading to the elimination of paternal mitochondria and the transmission of paternal chloroplast. The mechanisms involved would act during the final stages of pollen development, fertilisation or zygote development. Further experiments have to be performed to find out the mechanisms of paternal chloroplast transmission and maternal mitochondria elimination within the genus *Actinidia*.

Finally, the evolutionary implications of this unusual and contrasted inheritance for the two organelles in this dioecious species should also be investigated. Indeed, among land plants, only conifers, which are usually hermaphrodite species, share this particular mode of inheritance (Birky 1995).

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Commentaires critiques

Les investigations conduites dans divers groupes de plantes montrent que plusieurs mécanismes cytologiques ou moléculaires (amplification, exclusion ou dégradation) intervenant à des stades plus ou moins précoces de la reproduction sexuée (gamétogenèse, fécondation, embryogenèse) conditionnent le mode d'hérédité des organites (revue dans Sears 1980; Birky 1995). Chez le kiwi, les contenus en ADN de chacun des deux organites évoluent de manière opposée juste après la première division mitotique du grain de pollen. Le contenu en ADNcp double à l'intérieur de la cellule générative alors que celui de l'ADNmt chute (Nagata *et al.* 1999).

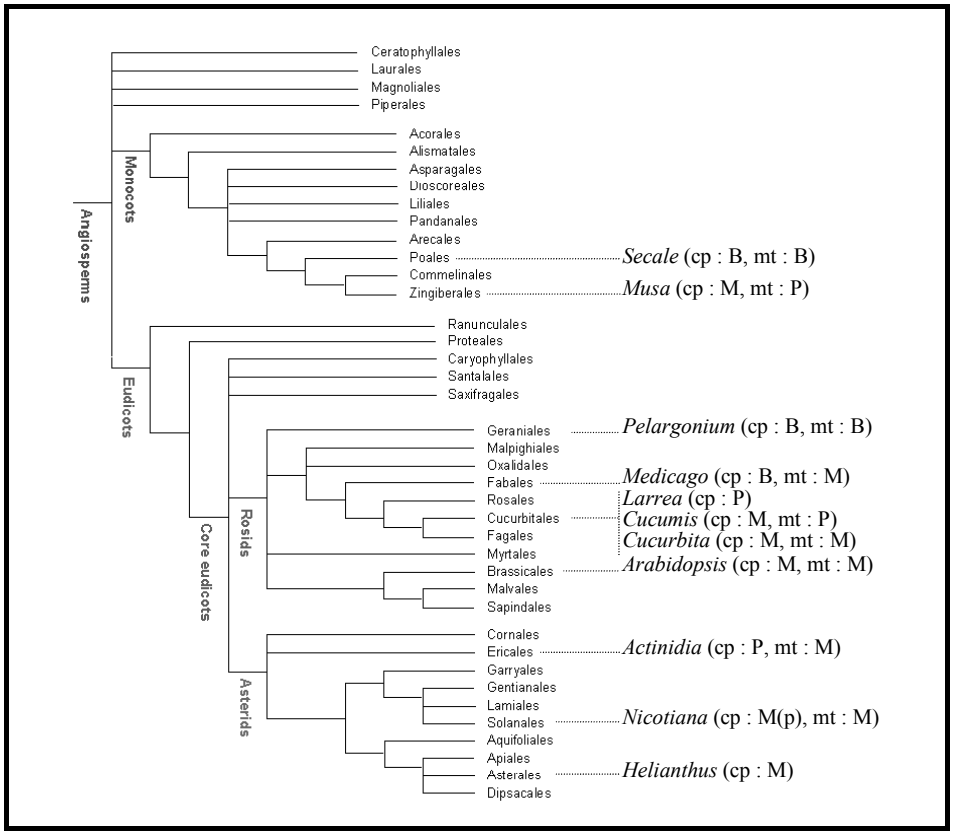

Figure 3 : Arbre phylogénétique des angiospermes (APG 1998) sur lequel est figuré le mode d'hérédité des organites cytoplasmiques (M : maternelle, P : paternelle, B : biparentale). Le mode d'hérédité est connu pour de nombreux taxa, la liste qui suit n'est pas exhaustive : *Actinidia* (Testolin et Cipriani 1997); *Arabidopsis* (Röbbelen 1966; Martinez-Zapater *et al.* 1992); *Cucumis* et *Cucurbita* (Havey *et al.* 1998); *Helianthus* (Rieseberg *et al.* 1994); *Larrea* (Yang *et al.* 2000); *Medicago* (Lee *et al.* 1988; Forsthoefel *et al.* 1992); *Musa* (Fauré *et al.* 1994); *Nicotiana* (Medgyesy *et al.* 1986); *Pelargonium* (Baur 1909; Nagata *et al.* 1999); *Secale* (Soliman *et al.* 1987);.

Le mode d'hérédité des organites a changé plusieurs fois au cours de l'évolution des végétaux (Birky 1995). Le placement du mode

d'hérédité des organites de certains taxons sur l'arbre phylogénétique des angiospermes (APG 1998) révèle que ce caractère est fortement homoplasique (Figure 3), ce qui n'est pas surprenant quand on connaît la diversité des mécanismes pouvant être impliqués dans son contrôle (Sears 1980; Hagemann et Schröder 1989).

Depuis les études conduites sur *Actinidia* entre 1995 et 1999, une autre angiosperme phylogénétiquement éloignée des *Actinidia*, le genre *Larrea* (Zygophyllaceae), a révélé le même mode d'hérédité paternelle stricte des chloroplastes (Yang *et al.* 2000). Dans les deux cas, ce sont des marqueurs moléculaires et non phénotypiques qui ont servi de base à l'établissement du mode de transmission. Lorsque le tri dans la descendance peut s'opérer visuellement, les effectifs pris en compte peuvent être plus importants, plusieurs centaines chez *Medicago* (Smith 1989) et plusieurs milliers chez *Cryptomeria* (Ohba *et al.* 1971), et on augmente alors la probabilité de détecter des événements de transmission atypique. Néanmoins, avec 262 descendants contrôlés (99 issus de croisements interspécifiques et 143 issus de croisements intraspécifiques) contre 20 issus de croisements interspécifiques pour *Larrea*, le genre *Actinidia* reste à ce jour le cas le mieux documenté d'hérédité chloroplastique paternelle stricte chez les angiospermes.

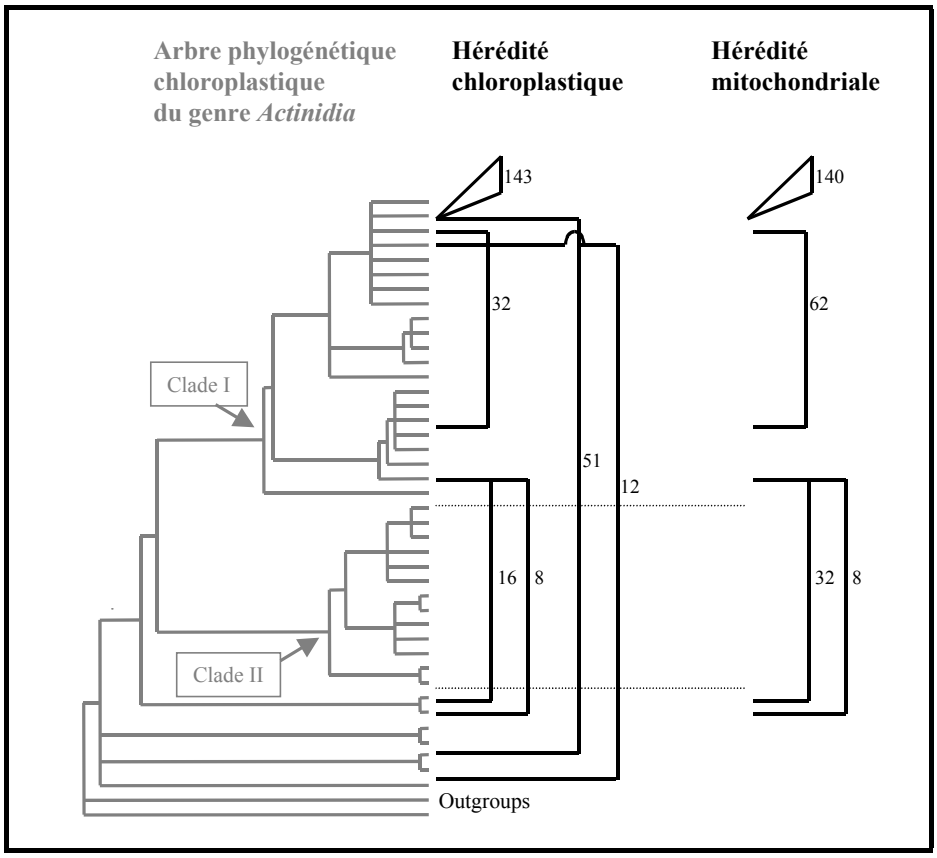

Figure 4 : Représentation sur l'arbre phylogénétique chloroplastique (cf. article 4) des croisements et des effectifs étudiés pour démontrer l'hérédité des organites chez *Actinidia*.

Le nombre et la diversité des croisements ainsi que l'effectif des descendances exploitées pour démontrer l'hérédité des organites chez *Actinidia* suggère que le mode d'hérédité contrastée des organites (paternelle pour l'ADNcp et maternelle pour l'ADNmt) soit homogène au sein du genre (Figure 4). Les taxons impliqués dans les croisements étudiés sont bien répartis sur l'arbre phylogénétique chloroplastique du genre *Actinidia* : ils appartiennent à la même espèce ou non, sont proches ou distants phylogénétiquement, se trouvent en position basale ou non sur l'arbre, jouent alternativement le rôle de parent mâle ou de parent femelle. D'autres croisements informatifs impliquant le clade II mériteraient cependant d'être étudiés. Une étude sur les deux autres genres de la famille des Actinidiaceae, i. e. *Saurauia* et *Clematoclethra*, permettrait de dire s'il s'agit d'un caractère affectant la famille toute entière.

2

A case of chloroplast heteroplasmy in kiwifruit (*Actinidia deliciosa*) that is not transmitted during sexual reproduction

J. Chat, S. Decroocq, V. Decroocq, R. J. Petit
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Résumé

Nous reportons ici le premier cas de chimère plastidique au sein du genre *Actinidia* où l'hérédité du génome chloroplastique est réputée paternelle. L'hétérogénéité de l'ADNcp observée au sein du cultivar hexaploïde 'D uno' porte sur la présence ou l'absence d'un site de restriction particulier *Msp*I dans une région comprise entre le gène *psb*C et le gène tRNA-Ser(UGA). L'hétérogénéité a été initialement observée en utilisant le polymorphisme de longueur des fragments de restriction et a été ensuite confirmée par clonage et séquençage. L'analyse des fragments clonés révélait la présence de deux haplotypes : le type le plus fréquent était présent dans 123 (88.5%) des 139 colonies étudiées. Le séquençage partiel des fragments *psb*C-*trn*S des deux haplotypes a révélé que le polymorphisme touche la région codante du gène *psb*C et consiste en une transition synonyme. Un croisement sans contamination impliquant 'D uno' en tant que parent mâle produit des plants porteurs de l'haplotype paternel le plus fréquent, indiquant soit un avantage sélectif en défaveur du type le plus rare ou, plus probablement, la fixation du type le plus fréquent dans les tissus produisant les gamètes. Les profils de restriction *Msp*I obtenus sur divers tissus suggèrent que le type le plus rare soit absent de la couche histogénique LII et que 'D uno' est une chimère plastidique de type péricline.

Préambule

Les *chimères*, *mosaïques* génétiques où deux lignées cellulaires distinctes génétiquement coexistent dans le méristème, sont connues depuis longtemps puisqu'un hybride de greffe entre une orange amère et un citronnier est signalé dès le XVII^{e} siècle. Toutefois, il faudra attendre le XX^{e} siècle pour que les bases cytologiques de l'état chimérique soient éclaircies. Le caractère *panaché*, exploité largement en horticulture ornementale, ne fut attribué à un état chimérique qu'à la suite des travaux de Baur (1909). Le lien entre déficience chlorophyllienne, *panachure*, hérédité biparentale non mendélienne des chloroplastes, *hétéroplasmie*, *ségrégation végétative*, et *chimère plastidique* devint alors évident, même si la terminologie utilisée diffère de celle d'aujourd'hui. Les bases théoriques de la génétique des organites cytoplasmiques sont donc posées, près de 50 ans après celles de la génétique nucléaire par Mendel.

Les chimères plastidiques existent dans de nombreux groupes de végétaux, comme en témoignent les panachures chez les gymnospermes (Ohba *et al.* 1971) mais aussi chez les angiospermes, monocotylédones (Stewart et Dermen 1979) comme dicotylédones (Stewart et Dermen 1975). Le caractère panaché résulte de la mutation d'un gène codant pour une protéine essentielle à la photosynthèse, empêchant le chloroplaste de jouer son rôle d'usine énergétique. Cette inaptitude se traduit phénotypiquement par une décoloration des cellules chlorophylliennes, le jaunissement est complet et létal lorsque la plante est *homoplasmique* ou incomplet (panachure) lorsque la plante est *hétéroplasmique* (polymorphisme chloroplastique intracellulaire ou intraplante). Même si le caractère panaché des feuilles a été signalé chez un grand nombre de végétaux, son apparition reste un événement rare et transitoire dans la nature.

Les panachures représentèrent pendant longtemps les seuls cas connus de polymorphisme chloroplastique intraplante mais aussi intraspécifique. Facilement identifiables micro- et macroscopiquement, elles ne devaient logiquement représenter que la partie visible de l'iceberg. Avec le développement croissant des outils de biologie moléculaire, on peut s'attendre à voir émerger de nouveaux cas de chimères plastidiques, révélant la partie jusqu'à présent cachée de l'iceberg (mutation silencieuse, mutation touchant une région non codante). On peut également s'attendre à ce que le postulat selon lequel le niveau de polymorphisme infraspécifique du génome chloroplastique est nul ou négligeable, déjà bien mis à mal ces dernières années (Harris et Ingram 1991), soit complètement battu en brèche.

L'hétéroplasmie et son stade ultime, la chimère plastidique, peuvent résulter de l'apparition *de novo* d'une mutation chloroplastique, de la transmission par un gamète d'un état

hétéroplasmique préexistant ou de la transmission d'un haplotype chloroplastique différent par chacun des deux gamètes. Le taux de substitution du génome chloroplastique est connu pour être faible, mais d'autres types de mutations plus fréquentes l'affectent (ex. microsatellites chloroplastiques) qui pourraient être à l'origine d'un polymorphisme intraplante (Doyle *et al.* 1998). De même, l'état hétéroplasmique est réputé transitoire, mais une hétéroplasmie stable par voie sexuée et asexuée a déjà été signalée chez les végétaux (Lax *et al.* 1987; Frey 1999). Malgré cela, l'hérédité biparentale reste fréquemment invoquée pour expliquer l'émergence de cas d'hétéroplasmie plastidique dans le règne végétal et c'est une des raisons pour laquelle nous nous y sommes intéressés dans le cadre de la thèse.

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Commentaires critiques

Lors de cette étude, l'hétéroplasmie soupçonnée par PCR-RFLP est bien confirmée par clonage et séquençage. Par contre, certaines incertitudes n'ont pu être levées :

- Les profils de restriction de divers types d'organes et de la descendance (lignée germinale issue de la LII) suggèrent qu'il s'agit d'une *chimère péricline* avec la couche LI (superficielle) présentant l'haplotype le plus rare, mais la preuve irréfutable n'est pas apportée. S'il est exclu que la chimère soit de type sectoriel, la composition des couches telle que suggérée dans l'article reste plus discutable. Compte tenu de la nature de la mutation, la culture *in vitro* (callogenèse et embryogenèse somatique) de tissus paraît plus indiquée pour confirmer la nature de l'état chimérique que des techniques d'hybridation *in situ*. C'est ce qui a été appliqué avec succès sur des chimères plastidiques (Fitter et Rose 1993) ou nucléaires (McPheeters et Skirvin 1983; Franks *et al.* 2002).
- Les deux haplotypes chloroplastiques présents chez D uno se distinguent par une substitution et existent à l'état homoplasmique au sein de l'espèce. Comme le taux de substitution du génome chloroplastique est très faible, une même substitution apparaissant à deux reprises au sein d'une espèce paraît hautement improbable et l'hypothèse d'une mutation *de novo* peut être écartée au profit de celle plus plausible de l'hérédité biparentale. Toutefois, la preuve irréfutable n'a pu être apportée compte tenu de l'absence de généalogie connue pour la plante en question. Seule une étude à grande échelle permettrait de dire si, et à quelle fréquence, des événements rares de type fuite maternelle (*maternal leakage*) peuvent se produire chez *Actinidia*.

Même s'il n'est pas exceptionnel de mettre en évidence des cas d'hétéroplasmie, notamment lors d'étude de phylogénie moléculaire à des fins de systématique (p. ex. Cros 1994), rares sont les publications qui en font état. Ceci est lié au fait que les endonucléases sont de plus en plus délaissées au profit du séquençage pour les études de phylogénie et que, dans ce contexte, la présence de deux formes moléculaires distinctes passe le plus souvent inaperçue (Boursot et Bonhomme 1986). Encore plus rares sont les travaux qui vont jusqu'à soupçonner la nature chimérique des plantes hétéroplasmiques.

Ce n'est pas la première fois qu'un polymorphisme uniquement décelable au niveau moléculaire permet de mettre en évidence une hétéroplasmie. On rappellera la démonstration déjà ancienne de l'hétéroplasmie chez *Medicago* où le chloroplaste se transmet biparentalement (Johnson et Palmer 1989; Fitter *et al.* 1996). Ce n'est

pas la première fois non plus qu'un polymorphisme décelable uniquement au niveau moléculaire sert à identifier un état chimérique. On signalera la description récente de deux chimères périclines chez la vigne à l'aide d'un locus nucléaire microsatellite (Franks *et al.* 2002). Mais, à notre connaissance, c'est la première fois qu'un polymorphisme décelable uniquement au niveau moléculaire est utilisé pour mettre en évidence une chimère plastidique.

3

A one-step organelle capture: gynogenetic kiwifruits with paternal chloroplasts

Chat J., S. Decroocq, R. J. Petit
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Résumé

Il a été précédemment montré que l'androgenèse, le développement d'un embryon haploïde à partir d'un noyau mâle, aboutit à un découplage instantané de la transmission des génomes nucléaires et cytoplasmiques (avec le génome nucléaire provenant du seul parent mâle et les génomes des organites du seul parent femelle). Nous rapportons ici pour la première fois un découplage résultant de la gynogenèse chez *Actinidia deliciosa* (le kiwi), une espèce végétale connue pour son mode d'hérédité chloroplastique paternelle. Suite à l'irradiation du pollen, la transmission des gènes nucléaires du parent mâle vers la descendance est inhibée, mais pas celle du génome chloroplastique. Cela démontre que les plastes peuvent être libérés du tube pollinique dans l'œuf en dépit d'une transmission nulle ou quasi-nulle des gènes nucléaires paternels. De tels événements d'hérédité opposée des génomes des organites et du noyau doivent rester rares dans la nature et sont peu susceptibles de mettre en danger la stabilité à long terme de l'association entre les différents génomes de la cellule. Néanmoins, ils peuvent conduire à des incongruences entre les arbres de gènes des organites et les arbres des espèces et pourraient constituer une alternative au scénario d'hybridation/introgression fréquemment invoqué pour rendre compte de telles incongruences.

Préambule

Chaque groupe constitué d'organismes vivants a connu une seule histoire vraie, inconnue mais néanmoins unique et certaine. En systématique moléculaire, il était classique de reconstruire l'histoire évolutive d'un groupe à partir d'un seul jeu de données en échantillonnant un seul taxon par espèce. On se basait alors sur deux postulats : l'espèce est reproductivement isolée et la variabilité intraspécifique, lorsqu'elle existe, est négligeable en regard de la variabilité interspécifique. Ces deux hypothèses ont fréquemment été battues en brèche et il est aujourd'hui conseillé de disposer (1) de plusieurs arbres phylogénétiques générés à partir de jeux de données distincts (caractères morphologiques, séquences chloroplastiques, séquences nucléaires) et (2) de prendre en compte la diversité intraspécifique lorsqu'elle existe. Il est alors fréquemment apparu que deux taxons proches sur l'arbre phylogénétique "nucléaire" ou "morphologique" peuvent se retrouver éloignés sur l'arbre phylogénétique "chloroplastique", et *vice versa* (Figure 5).

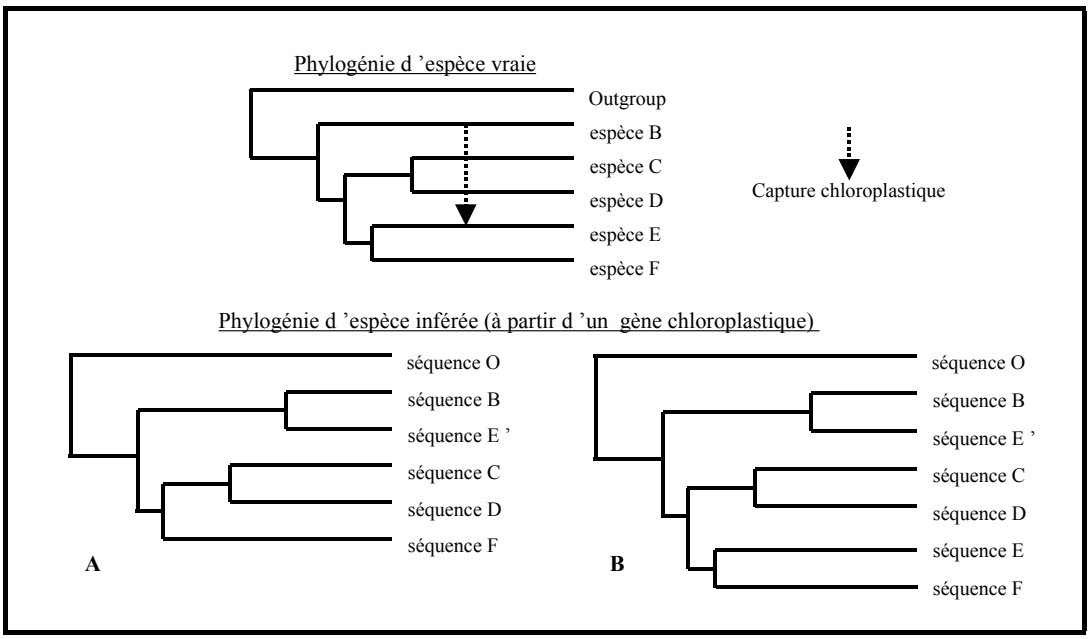

Figure 5 : La capture chloroplastique en systématique moléculaire. Phylogénie d'espèce inférée à partir d'un gène chloroplastique après introgression du chloroplaste de l'espèce B dans l'espèce E, suivie (A) ou non (B) de l'extinction de la lignée correspondante au chloroplaste E (d'après Doyle 1992).

Un flux de gènes cytoplasmiques accompagné d'une apparente absence de flux de gènes nucléaires est traditionnellement rendu responsable de telles incongruences (revue dans Rieseberg et Soltis 1991; Doyle 1992). Les systématiciens emploient le terme de *capture chloroplastique* pour désigner le "remplacement" du cytoplasme natif d'un taxon par un cytoplasme étranger. Lorsqu'il a pu être établi avec certitude (p. ex. Rieseberg *et al.* 1990; Soltis *et al.* 1991), le scénario

de capture chloroplastique débute toujours par un premier événement d'hybridation entre deux espèces, suivi par des croisements successifs en retour avec une des deux espèces parentes (mâle pour la majorité des angiospermes du fait de l'hérédité maternelle des organites). On aboutit alors à une dilution progressive du fond génétique nucléaire de l'espèce ayant "donné" son cytoplasme.

Même si le terme de capture cytoplasmique est très parlant pour celui qui manipule des arbres phylogénétiques d'espèces, il concourt à donner une image inexacte des processus biologiques sous-jacents. Plaçons-nous dans le cas des angiospermes où l'hérédité chloroplastique strictement maternelle est prédominante. Deux cas peuvent se présenter selon que l'événement migratoire est un transport de graine (Rieseberg et Soltis 1991) ou de pollen (Potts et Reid 1988) (Figure 6). Dans le premier cas, on assiste à l'introgression des organites cytoplasmiques de l'espèce exotique dans l'espèce indigène ("chloroplast capture" au sens littéral du terme), alors que dans le deuxième, c'est l'espèce indigène qui cède son cytoplasme à l'espèce exotique ("*pollen swamping*", mais le terme de "capture nucléaire" pourrait aussi convenir).

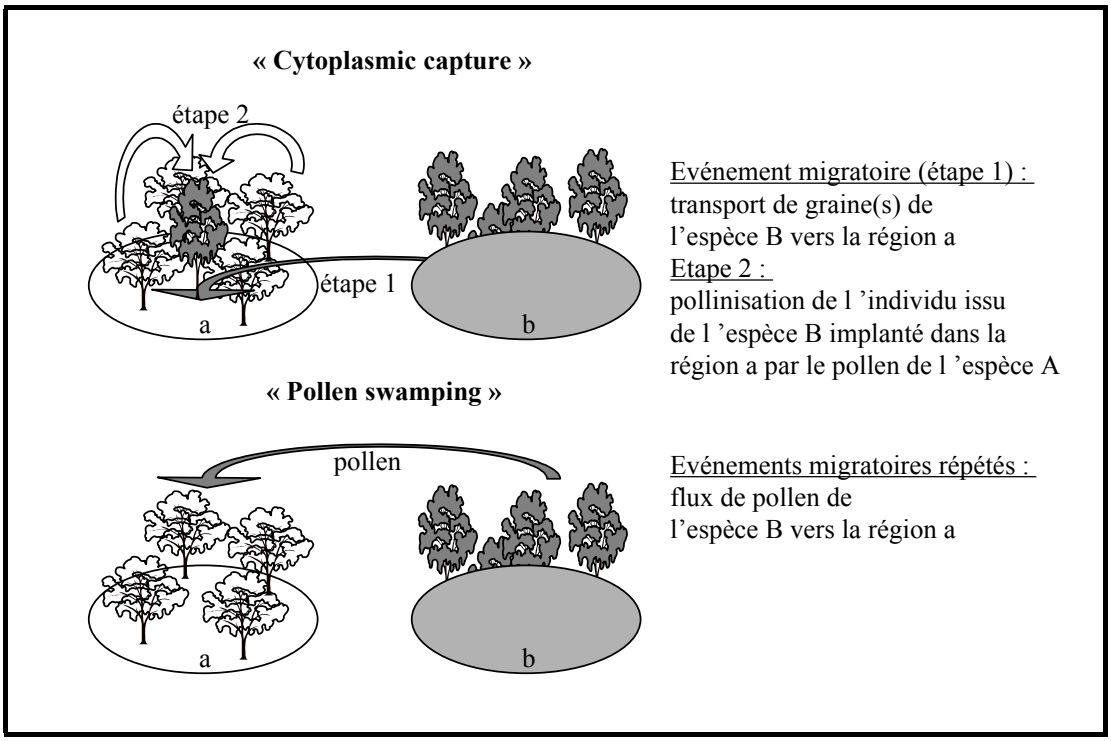

Figure 6 : Les processus biologiques et écologiques à l'origine d'une capture chloroplastique (espèce A indigène de l'aire géographique a représentée en blanc, espèce B indigène de l'aire géographique b représentée en gris).

Dans les deux cas, plusieurs rétrocroisements sont nécessaires avant que le processus de capture chloroplastique au sens des systématiciens moléculaires soit achevé. Ceci tient à une des caractéristiques propres à la reproduction sexuée qui veut que, quel

que soit leur mode d'hérédité, les génomes cytoplasmiques ne se transmettent jamais isolément mais toujours accompagnés par le noyau, généralement réduit après méiose, afin que chacun des deux partenaires mâle et femelle fournisse la moitié des gènes nucléaires à la génération diploïde suivante.

Existe-t-il des modes de reproduction qui font exception à cette règle ? Dans l'affirmative, on peut imaginer un scénario de capture chloroplastique instantanée. Voilà la raison pour laquelle nous nous sommes intéressés de si près aux modes de reproduction parthénogénétique.

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A one-step organelle capture: gynogenetic kiwifruits with paternal chloroplasts

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Androgenesis, the development of a haploid embryo from a male nucleus, has been shown to result in the instantaneous uncoupling of the transmission of the organelle and nuclear genomes (with the nuclear genome originating from the male parent only and the organelle genomes from the female parent). We report, for the first time,●●2●● uncoupling resulting from gynogenesis, in *Actinidia deliciosa* (kiwifruit), a plant species known for its paternal mode of chloroplast inheritance. Following pollen irradiation, transmission of nuclear genes from the pollen parent to the progeny was inhibited, but transmission of the chloroplast genome was not. This demonstrates that plastids can be discharged from the pollen tube into the egg with little or no concomitant transmission of paternal nuclear genes. Such events of opposite inheritance of the organelle and nuclear genomes must be very rare in nature and are unlikely to endanger the long-term stability of the association between the different genomes of the cell. However, they could lead to incongruences between organelle gene trees and species trees and may constitute an alternative to the hybridization/introgression scenario commonly invoked to account for such incongruences.

Keywords: gynogenesis; paternal chloroplast inheritance; phylogeny; incongruence; species tree

1. INTRODUCTION

Two of the three genomes found in eukaryotic plant cells, the chloroplast and mitochondrial genomes are ancient free-living eubacteria now forming obligatory dependent relationships with the nucleus (Margulis 1970; Gray 1999). The long-term stability of such an association requires that the interests of the symbionts align with those of the host (Herre *et al.* 1999). In the course of evolution, the mitochondrial and chloroplast genomes have undergone massive losses of functional genes, to the point that they can no longer resume an independent existence (Maynard-Smith & Szathmary 1997; Gray 1999). Nevertheless, situations likely to generate conflicts of interest between the organelle and nuclear genomes still exist due to their contrasting mode of transmission (Eberhard 1980; Cosmides & Tooby 1981; Moran & Wernegreen 2000). The complete uncoupling of the transmission of organelle genomes from that of the nuclear genome (whereby an organelle genome is transmitted from one parent and the nuclear genome from the other parent) should, in principle, generate even more drastic genomic conflicts, potentially endangering the stability of the association and hence the very existence of the eukaryotic cell. Despite their potential interest, cases of transmission of organelle and nuclear genomes through opposite sexes have not attracted the interest of evolutionists, probably because reports of their occurrence remain extremely rare. In fact, the only well-known cases of transmission of different genomes through opposite sexes do not involve cytonuclear transmission but involve, instead, chloroplast and mitochondria, when one organelle is paternally inherited and the other maternally inherited as found, in particular, in some conifers (Wagner *et al.* 1987; Neale & Sederoff 1989). However, the prospects for the uncoupling of organelle transmission from that of the nucleus do exist. First, under artificial conditions, intact organelles can be cloned into donor cells of other individuals from the same species in the absence of nuclear transfer in plants (Verhoeven & Blass 1988; Eigel & Koop 1992) as well as in animals (Pinkert *et al.* 1997), including humans (Cohen *et al.* 1997). Second, there are reproductive processes that mimic sexual reproduction but omit syngamy (Kimber & Riley 1963; Nogler 1984), leading to the uniparental transmission of the nuclear genome, another prerequisite for such uncoupling.

In apomictic plants that reproduce asexually through seeds, embryos inheriting their diploid nucleus from one parent and their organelle genomes from the other parent have not yet been reported. In such species, found in some 40 angiosperm families (Vielle Calzada *et al.* 1996) not only meiosis but also fertilization is by-passed, giving birth to individuals that are the exact genetic copy of the seed parent (Nogler 1984; Asker & Jerling 1992) or, as recently discovered in a rare *Cupressus* species, of the pollen parent (Pichot *et al.* 2001). In maternal apomixis, even if the development of the seed is sometimes dependent on the fertilization of the polar nuclei for endosperm development, the male parent does not contribute at all to the genetic constitution of the embryo. Similarly, and quite strikingly, in *Cupressus*, the only case of paternal apomixis reported so far, the cytoplasm of the embryo is expected to be male in origin, like the nuclear genome, since both chloroplast and mitochondrial genomes are paternally inherited in *Cupressaceae* (Chesnoy 1987; Neale *et al.* 1991).

In androgenetic plants, however, haploid embryos are produced that originate from the development of a male gamete within the embryo sac of the female parent,

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resulting in uniparental paternal inheritance of the nuclear genome, whereas the organelle genomes are still contributed by the female parent. This results, therefore, in the complete uncoupling of the transmission of organelle and nuclear genomes. Androgenetic plants, although considerably rarer than apomictic plants and, to a lesser extent, than gynogenetic plants (i.e. haploid plants deriving from the development of the female gamete), have been reported several times since the early twentieth century (e.g. Clausen & Lammerts 1929; Kehr 1951; Burk 1962; Singh & Cornu 1976). They are known to occur spontaneously, at a low frequency, in various angiosperms belonging to both mono- and dicotyledons, for example, *Capsicum* (Campos & Morgan 1958), *Nicotiana* (Burk 1962), *Petunia* (Singh & Cornu 1976) and *Zea* (Chase 1963). In the 1960s, Goodsell (1961) and Chase (1963) demonstrated that maize androgenetic haploids had the organelle genomes of their mother, together with the haploid nucleus of their father. In the 1980s, the same conclusion was reached for *Nicotiana* and *Petunia* using molecular markers (Pelletier *et al.* 1987; Raquin *et al.* 1989; Horlow *et al.* 1993).

By contrast, no case of gynogenesis leading to the uncoupling of the transmission of nuclear and organelle genomes has yet been reported. In principle, it is conceivable that a male plant could contribute its chloroplasts or mitochondria to a gynogenetic offspring, provided that paternal or biparental inheritance of the organelle genomes can occur in that species. Paternal inheritance of the chloroplast genome has been demonstrated in the genus *Actinidia* (Cipriani *et al.* 1995; Chat *et al.* 1999). Furthermore, there have been successful attempts to produce *Actinidia* haploids using irradiated pollen (Pandey *et al.* 1990; Chalak & Legave 1997), a physical treatment well known to trigger haploidization processes (Lacadena 1974). In particular, four trihaploids were recovered from an experiment conducted in the cultivated hexaploid species *A. deliciosa* (Chalak & Legave 1997). In the present study, we examined whether the chloroplast genome of the pollen parent had been transmitted and checked if only the female parent had contributed nuclear genes to this haploid progeny using several codominant nuclear markers (Weising *et al.* 1996; Huang *et al.* 1998). In conjunction with previous reports of androgenesis, our findings raise the question of whether cases of complete uncoupling of the transmission of organelle and nuclear genomes occur in nature, for instance following interspecific hybridization. Such whole-genome horizontal transfers could explain some of the incongruences between organelle gene trees and species trees in plants and should improve our understanding of the evolutionary relationships between organelle and nuclear genomes.

2. MATERIAL AND METHODS

(a) *Plant material*

Kiwifruit is a dioecious species; the two hexaploid parents used in this study were 'D uno', a male plant, and 'Hayward', a female cultivar. In 1994, Hayward was pollinated with irradiated pollen from D uno to induce gynogenesis (Chalak & Legave 1997). Several seedlings resulting from that experiment were assessed for ploidy. Four of them were trihaploids, as determined by flow cytometry (Chalak & Legave 1997). These were designated H11, H12, H13 and H14. A shoot resulting from the spontaneous chromosome doubling of H14 during *in vitro* adventitious regeneration was also propagated and designated DH14. Forty full sibs originating from the controlled cross Hayward × D uno performed with non-irradiated pollen were also included in the study. The two parents, the four trihaploids, the doubled trihaploid and the 40 full sibs were analysed using molecular markers. Total genomic DNA was isolated from leaf tissue according to a modified CTAB●●3●● procedure (Chat *et al.* 1999). All the DNA extracts were diluted to a final concentration of 5 ng μl^{-1} to be used as a template for PCR amplification.

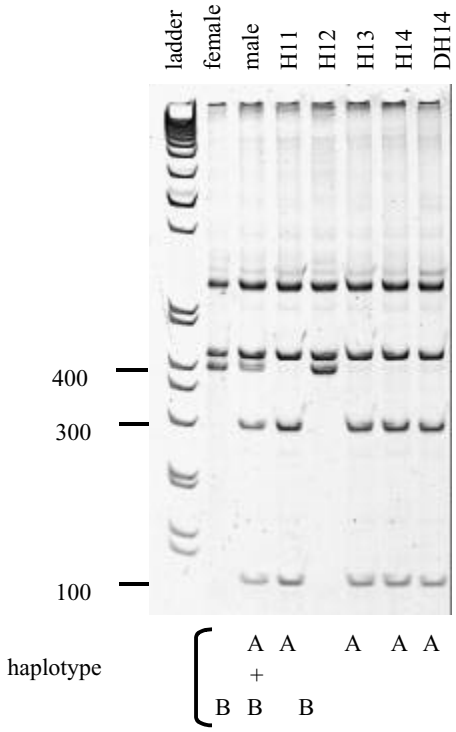

Figure 1. Inheritance of cpDNA in the four trihaploids (H11, H12, H13 and H14) and in the doubled trihaploid (DH14) resulting from H14. The *psb*C-*trn*S cpDNA fragment amplified by PCR (1600 bp) is digested by the *Msp*I restriction enzyme. The fragment sizes (in bp) for the type A and type B specific bands are indicated on the left. Ladder: 1 kb molecular weight marker.

(b) *Polymorphism of the chloroplast genome*

The chloroplast DNA (cpDNA) polymorphic region analysed is located between the *psb*C and *trn*S genes, coding for a *ps*II protein and the tRNA-Ser(UGA), respectively. Universal primers (Demesure *et al.* 1995) were used to amplify this particular cpDNA region. Two different cpDNA haplotypes, called haplotypes A and B, are present within the species *A. deliciosa* (Chat *et al.* 1999). Haplotype B differs from haplotype A by an extra *Msp*I recognition site within the *psb*C-*trn*S region. The pollen parent D uno possesses both haplotypes (heteroplasmic state) whereas the female parent Hayward possesses only haplotype B (homoplasmic). As demonstrated in a previous study, the heteroplasmic state of D uno (responsible for the restriction pattern observed: two strong bands of 300 and 100 bp, specific to haplotype A, together with the faint 400 bp band specific to haplotype B; see figure 1) is actually due to its periclinal plastid chimeric state. The second histogenic layer (LII) of D uno contains exclusively plastids of type A, and as a consequence only this

type is transmitted to the next generation during sexual reproduction (Chat *et al.* 2002). This polymorphism was used to infer the plastid origin of the four trihaploids and the doubled trihaploid. Conditions for DNA amplification and electrophoresis have been described previously (Chat *et al.* 1999).

(c) *Polymorphism of the nuclear genes*

Nuclear composition of the trihaploids was investigated using five microsatellite (SSR) primer pairs, UDK96-001, UDK96-030 (Huang *et al.* 1998) and 721, 722, 735 (Weising *et al.* 1996). As the primer pairs all revealed differences between the two parents, they were subsequently used to determine the genotype of the four trihaploids and that of the doubled trihaploid. Conditions for SSR amplification and electrophoresis have been detailed previously (Chat *et al.* 2002).

(d) *Statistical analysis of the SSR data*

Owing to the difficulty of distinguishing between one-dose versus multiple-dose allele conditions within the segregating populations, SSR analysis was performed based on the presence [+] versus the absence [−] of the allele. Chi-square tests were used to determine goodness-of-fit of the segregation ratios among the 40 sexual hybrids. The inheritance of nuclear SSR markers is of polysomic type (see § 3), a finding that is in agreement with the conclusion of Testolin & Ferguson (1997) that *A. deliciosa* is an autohexaploid. As a consequence, for markers that are only present in the male parent, the probability of allele presence [+] in the male gamete corresponds to random sampling without replacement of three alleles among the six paternal ones. In the male gamete population, ratios [+] : [−] of 1 : 1, 4 : 1, 19 : 1 are expected for alleles in simplex, duplex, triplex conditions in the male parent, whereas alleles present in four copies or more in the male parent should be present in at least one copy in all male gametes produced. Considering all loci, we then calculated the probability: (i) for a zygote (2n = 6x) to inherit at least one paternal-specific allele; and (ii) for a haploid (n = 3x) to receive none of the paternal-specific allele, assuming random elimination of three alleles among the six initially present in the zygote.

3. RESULTS

(a) *Chloroplast composition of the trihaploids*

Three out of the four trihaploids had haplotype A only, indicating a paternal origin for their cpDNA genome (figure 1). By contrast, H12 exhibited haplotype B only. The presence of haplotype B (identical to the chloroplast genome of the mother) in one of the four trihaploids may be explained by occasional maternal inheritance of the cpDNA, possibly due to paternal cpDNA damage caused by irradiation, or by reorganization of the histogenic layer of the chimeric pollen parent D uno, leading to the presence of haplotype B in the LII layers of the father, followed by normal paternal inheritance of cpDNA (Chat *et al.* 2002).

(b) *Nuclear composition of the trihaploids*

The five SSR primer pairs selected produced 13 alleles specific to the male parent D uno. None of these male-specific alleles were transmitted to the trihaploids; instead, all the alleles of the trihaploids were of maternal origin (figure 2). As expected, the doubled trihaploid DH14 cannot be distinguished genetically from the trihaploid H14. Genotypes of both the parents and the trihaploids are summarized and compared with those of the full sibs in table 1. As previously reported in *Actinidia* (Huang *et al.* 1998), some of the SSR primer pairs produce more bands than expected given the ploidy level of the plant, suggesting that the locus has been duplicated at least once in the course of evolution. This was the case for locus 735 that was subsequently discarded from further statistical analyses. Among the 10 remaining paternal specific-alleles, simplex condition was the most common (table 1).

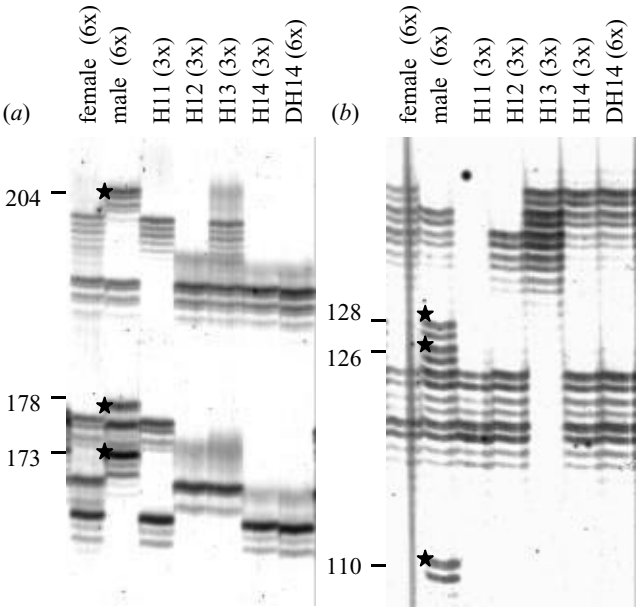

Figure 2. Segregation of nuclear SSR alleles among the four trihaploids (H11, H12, H13 and H14) and the doubled trihaploid (DH14) resulting from H14: PCR amplification of the SSR loci (*a*) 721 and (*b*) 722. Only the paternal-specific bands are indicated on the left margin by their fragment sizes (in bp) and on the picture by a star.

(c) *Statistical analyses of the SSR loci*

Chi-square tests indicated no segregation distortion among the 40 full sibs for the 10 alleles considered. This suggests that the allelic composition of both parents had been correctly inferred and that no bias had occurred during sexual transmission. All the SSR nuclear markers polymorphic enough to examine chromosomal pairing (UDK96-030, 721, 722) displayed a polysomic inheritance. Moreover, three of the SSR loci used in this study, i.e. UDK96-001, UDK96-030 and 721, are known to segregate independently (Testolin *et al.* 2001). It is likely that all four loci examined belong to different linkage groups owing to the high basic number of chromosomes reported in *Actinidia* (x = 29). Consequently, the probability to detect only genotypes [−] can be calculated as the product of the probability to be [−] at each of the four loci. The probability that none of the four trihaploids inherit any of the 10 paternal-specific nuclear SSR alleles is extremely low when assuming random elimination of three sets of chromosomes after syngamy (2.9×10^{-7}; details given in electronic Appendix A, available on The Royal Society's Publications Web site).

Table 1. Comparison of the nuclear allelic composition of the 40 full sibs population Hayward × D uno with that of the 4 trihaploids obtained from the same cross using irradiated pollen. Deviation from Mendelian segregation in the F1 tested using χ^2 values (n.s.: non-significant at $p = 0.05$). nd: not determined

	segregation ratio among							
	paternal-specific SSR alleles		40 hexaploid F1				4 trihaploids	
locus	allele size (bp)	allele dosage	genetic model ([+] : [−])	observed [+]	observed [−]	χ^2	observed [+]	observed [−]
UDK96-001	238	1	1 : 1	24	16	1.60 n.s.	0	4
UDK96-030	155	1	1 : 1	18	22	0.40 n.s.	0	4
UDK96-030	123	1	1 : 1	23	17	0.90 n.s.	0	4
UDK96-030	107	1	1 : 1	21	19	0.10 n.s.	0	4
721	204	1	1 : 1	17	23	0.90 n.s.	0	4
721	178	1	1 : 1	25	15	2.50 n.s.	0	4
721	173	2	4 : 1	28	12	2.50 n.s.	0	4
722	128	1	1 : 1	20	20	0.00 n.s.	0	4
722	126	1	1 : 1	19	21	0.10 n.s.	0	4
722	110	1	1 : 1	22	18	0.40 n.s.	0	4
735	186	nd	nd	14	26	nd	0	4
735	180	nd	nd	37	3	nd	0	4
735	94	nd	nd	36	4	nd	0	4

4. DISCUSSION

The results obtained in kiwifruit demonstrate that, despite pollen irradiation, the plastids from the male plant can still be transmitted to the egg cell of the female. Indeed, the fact that the cpDNA was of paternal origin in three trihaploids indicates that the release of sperm cells, or at least of some paternal cytoplasm, into the embryo sac had occurred for these individuals, whereas no nuclear gene transmission from the father was detected. The presence of maternal-specific nuclear alleles in the trihaploids led us to exclude androgenesis, whereas the absence of paternal-specific nuclear alleles in each of the four trihaploids allowed us to reject the hypothesis of random chromosome elimination after syngamy. Thus, the hypothesis that best fits with our experimental data is gynogenesis, caused by the absence of fertiliztion, or by syngamy followed by preferential elimination of the male chromosomes. Empirical arguments already exist that support either scenario. Since the 1970s, it has been established that haploids originating following congeneric (Subrahmanyam & Kasha 1973) and intergeneric hybridization (Barclay 1975) result from the fertilization of the egg cell, followed by the selective and gradual elimination of the pollen parent chromosomes during the first mitotic divisions of the embryo. Consequently, a strong bias in favour of the elimination of the paternal chromosomes is plausible here, particularly considering that the paternal chromosomes must have been damaged by irradiation. However, the finding of diploid (instead of triploid) endosperm following pollination with irradiated pollen in kiwifruit (Musial & Przywara 1999) could indicate that double fertilization has not occurred. Figure 3 summarizes the sexual reproduction in *Actinidia* and the two processes possibly involved in haploid formation. Irrespective of whether syngamy had occurred or not, our experimental data indicate that complete chloroplast replacement can be achieved within one generation in *Actinidia* in the absence of significant nuclear exchanges, following pollen irradiation.

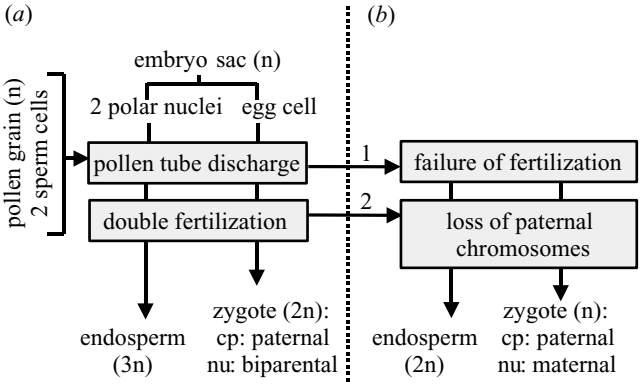

Figure 3. Possible mechanisms involved in haploid formation in kiwifruit, as compared with the typical sexual reproduction. (*a*) Sexual reproduction. After pollen deposition and germination on the stigma, the pollen tube grows down the style until it reaches the embryo sac (Hopping & Jerram 1979). It then releases two sperm nuclei: one fuses with the egg nucleus whereas the other fuses with the two polar nuclei to produce the 3n endosperm (Harvey & Fraser 1988). The 3n ploidy of the endosperm was deduced from cytological observations (Harvey & Fraser 1988) and further confirmed by chromosome counting (Machno & Przywara 1997). The embryo inherits the chloroplast genome from its father (Cipriani *et al.* 1995; Testolin & Cipriani 1997; Chat *et al.* 1999). (*b*) Haploid formation following pollen irradiation. Haploids result from gynogenesis caused by the absence of fertilization (pathway 1) or by elimination of paternal chromosomes following fertilization (pathway 2). Most, if not all, of the haploids inherit the chloroplast genome from their father. The endosperm (2n) develops autonomously (Musial & Przywara 1999). cp: chloroplast genome. nu: nuclear genome.

Since interspecific pollen (like irradiated pollen) can

stimulate the production of haploids, the occurrence of spontaneous haploids is conceivable in kiwifruit. In such cases, gynogenesis associated with paternal transmission of the chloroplast could lead to the instantaneous chloroplast transfer across species barriers and, hence, to the uncoupling of the transmission of the organelle genomes from that of the nuclear genome. This will result in new cytonuclear associations. For the organelles, such new cytonuclear associations could act as a bridge between two distinct host species provided two conditions are met: (i) the haploids produced must be fertile; and (ii) the haploids produced must be males (otherwise their plastids will not be transmitted further). In the genus *Actinidia*, there have already been reports of haploids following manual interspecific hybridization (Chat *et al.* 1996), in line with the observation that polyploid species are particularly prone to producing haploids (Kimber & Riley 1963). In addition, the production of unreduced gametes (Chat *et al.* 1996; Yan *et al.* 1997) or the spontaneous somatic doubling of the chromosomes (Chalak & Legave 1997) observed in *Actinidia* could restore the fertility of the haploids. However, it seems unlikely that a female plant would give rise to a male plant through gynogenesis due to the presence of sexual chromosomes, the male being the heterogametic sex in kiwifruit (Harvey *et al.* 1997*b*; Gill *et al.* 1998). Residing in a female plant, the captured chloroplast genome cannot normally invade the new taxon, since females do not transmit their chloroplast genome (Cipriani *et al.* 1995; Chat *et al.* 1999). However, rare cases of biparental chloroplast inheritance seem to occur in *Actinidia* (Chat *et al.* 2002). Furthermore, although considered to be ancestral in *Actinidia* (Harvey *et al.* 1997*a*), dioecy is sometimes relaxed in this genus (McNeilage 1991). These two facts could contradict our first expectations.

The possibility of chloroplast exchanges in the absence of significant nuclear gene flow must be considered when reconstructing species phylogenies, as a growing number of incongruences between chloroplast gene trees and species trees have been reported (reviewed by Rieseberg & Soltis 1991; Doyle 1992). The most frequently invoked scenarios involve a first event of interspecific hybridization followed by several successive backcrosses until the nuclear genome of the recipient species is completely replaced, a process called 'cytoplasmic capture' (reviewed by Rieseberg & Soltis 1991) or 'pollen swamping' (Potts & Reid 1988; Petit *et al.* 1997; Belahbib *et al.* 2001) (depending on the relative importance of pollen and seed flow and on whether species' ranges are considered to be dynamic). Another possibility involving hemigamy has been proposed for *Gossypium* (Wendel *et al.* 1991). Hemigamy is caused by an abnormal fertilization resulting in a chimeric embryo with haploid tissues of paternal and maternal origin (Turcotte & Feaster 1967). Androgenesis or gynogenesis associated with maternal or paternal inheritance of organelle genes could also be involved, as discussed above for *Actinidia*. In fact, only in these two cases could one speak of 'cytoplasmic capture' in its strictest sense, i.e., the instantaneous capture of an organelle genome of one individual by another one without concomitant transfer of nuclear genes.

Results such as those presented here for the kiwifruit are also relevant to the understanding of intergenomic conflicts and evolutionary interdependence of the organelle and nuclear genomes. Whereas the contrasting inheritance patterns between the organelle genomes, the sex chromosomes and the autosomes have already been identified as a source of genomic conflicts within eukaryotic organisms (Eberhard 1980; Cosmides & Tooby 1981; Maynard-Smith & Szathmary 1997), the question of the obligatory transmission of organelle genomes together with nuclear chromosomes across generation has not yet been explored, possibly because of the perceived absence of exceptions to this rule. Studies with plants and, to a lesser extent, animals (androgenesis has been reported in insects; Mantovani & Scali (1992); Tinti & Scali (1996), and molluscs; Komaru *et al.* (1998), and can be induced in fishes and molluscs; Corley-Smith & Brandhorst (1999)) indicate that the barriers preventing organelle genomes from being transmitted independently of the nuclear genome are not total and deserve to be studied, both empirically and theoretically. Such independent transmissions of the organelle genomes are expected to exacerbate already existing conflicts of interest between the organelle symbionts and the host cell, because horizontal transmission of whole organelle genomes to new hosts should favour their selfishness and their moves towards parasitism (Maynard-Smith & Szathmary 1997). Although horizontal transmissions of organelle genomes sometimes take place in nature through pseudo-sexual reproduction events, as discussed here for the kiwifruit, they seem to be kept at low levels and/or to lead to an evolutionary cul-de-sac (as when newly captured organelle genomes end up in the non-transmitting sex). This may reflect the fact that the nuclear genome should readily suppress selfish organelle genes owing to its disproportionately high number of loci, a phenomenon called 'central control' by Maynard-Smith & Szathmary (1997). In fact, endosymbiotic organelles have been considered to be 'encapsulated slaves' rather than mutualists, since the advantages of the relationship for the organelles are impossible to quantify in the absence of free-living stages (Douglas & Smith 1989). We argue that the extreme rarity of events whereby organelles are 'freely' transmitted (i.e. independently of the nucleus) reinforces this view.

The process described here is limited to transfers between taxa showing compatible nucleo–cytoplasmic interactions, that is, between closely related species (Grun 1976). Horizontal transfers of whole organelle genomes between more divergent lineages have been inferred in algae but they result from secondary endosymbioses. Such events are fundamentally different since they involve the capture of whole eukaryotic cells—instead of isolated organelles— followed by the progressive elimination of the endosymbiotic nucleus during evolution (McFadden *et al.* 1996).

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Commentaires critiques

Si les études phylogénétiques de genres sont nombreuses à invoquer une capture chloroplastique pour expliquer le partage d'un même haplotype chloroplastique par deux taxons morphologiquement éloignés, peu d'entre-elles recherchent des scénarios alternatifs à celui de l'hybridation/introgression. A notre connaissance, seule une étude chez *Gossypium* a, par le passé, envisagé l'éventualité d'un processus apomictique comme possible origine d'une capture (Wendel *et al.* 1991).

Autres pistes

Les seuls haploïdes gynogénétiques dont on disposait pour cette étude provenaient d'une expérience de gynogenèse induite par irradiation du pollen. Il aurait été intéressant de conduire le même type d'étude avec des haploïdes obtenus lors de tentatives d'hybridations interspécifiques, processus susceptible de se produire dans la nature lorsque deux espèces sont en contact.

Nous avons ici évoqué le cas de la capture chloroplastique lors de la gynogenèse chez le kiwi, nous pouvons également suspecter une capture mitochondriale lors de l'androgenèse. L'androgenèse *in situ* étant encore plus rare que la gynogenèse *in situ*, cette étude, aussi intéressante soit-elle, est encore plus difficile à envisager.

Dans les deux cas précédents, la capture chloroplastique et la capture mitochondriale, nous nous situons dans le cadre d'un découplage entre la transmission des organites et celle du noyau mais nous pouvons imaginer d'autres types de découplage qu'il serait intéressant d'examiner de plus près. L'existence de chromosomes sexuels n'est encore qu'hypothétique chez le kiwi, mais a été démontrée chez d'autres plantes (Richards 1997). Si son existence se confirme chez le kiwi, on s'attend à ce que le génome chloroplastique et le chromosome sexuel retracent la même histoire phylogénétique, celle de la lignée paternelle. Mais, du simple fait d'un phénotype sexuel labile, on peut imaginer de rares découplages entre le génome chloroplastique et le chromosome Y générateurs d'incongruences entre les deux phylogénies.

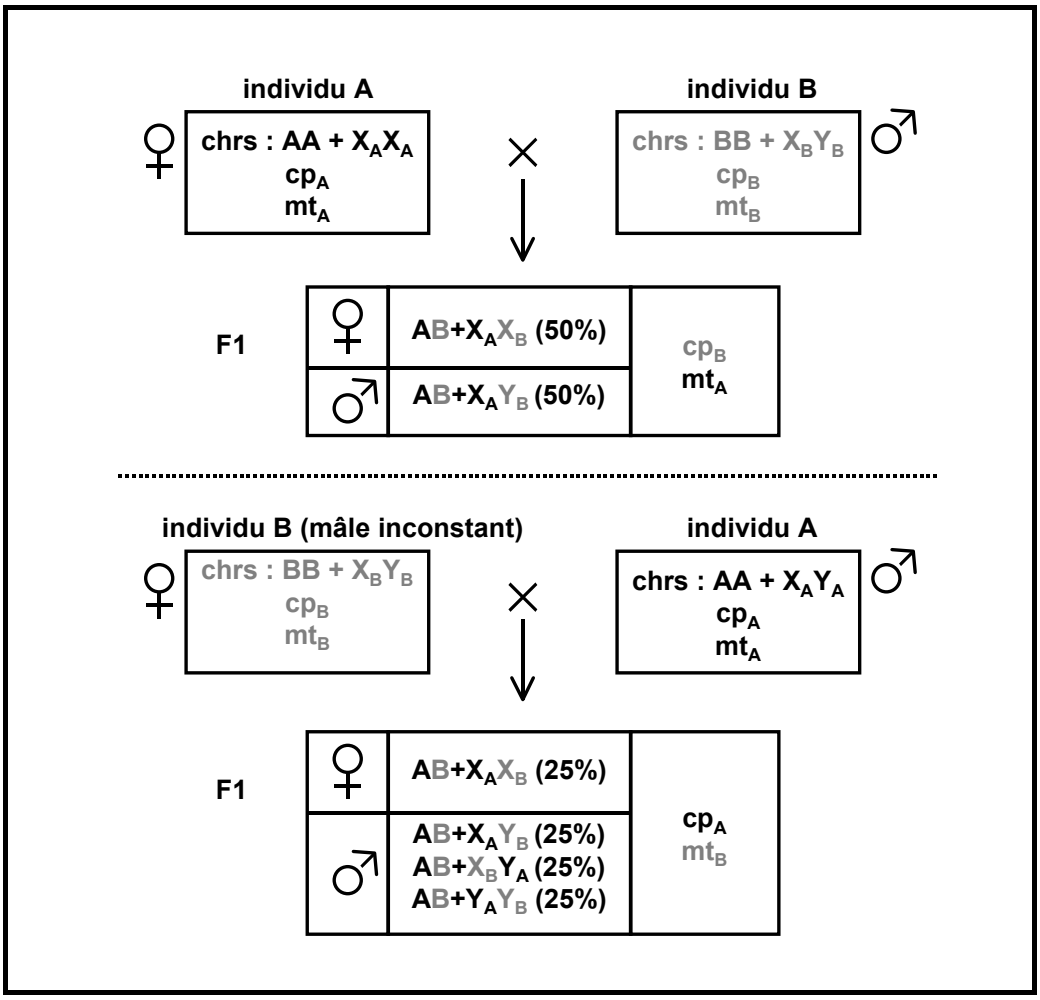

Figure 7 : Réassortiment du couple chromosome Y et génome chloroplastique par le biais des mâles inconstants. A et B désignent les autosomes et X et Y les chromosomes sexuels.

Le genre *Actinidia* (Ferguson 1990b) ainsi que *Saurauia* (Soejarto 1969) et *Clematoclethra* (Dickison 1972) sont dioïques, suggérant que ce caractère est commun à l'ensemble de la famille des Actinidiaceae. Cependant, en ce qui concerne les *Actinidia*, la dioécie n'est pas stricte (McNeilage 1991b; McNeilage et Steinhagen 1998). En effet, il arrive que certains plants mâles utilisés comme pollinisateurs en verger commercial soient porteurs de petits fruits contenant des graines, on les appelle des *mâles inconstants*. En autofécondation, ces mâles inconstants donnent une descendance composée d'une femelle pour trois mâles (Testolin *et al.* 1995). Un mâle inconstant serait donc susceptible de transmettre son chromosome Y sans transmettre son génome chloroplastique, provoquant une rupture dans la transmission couplée du chromosome Y et du génome chloroplastique (Figure 7). Le réassortiment entre ces deux éléments pourrait s'opérer dès la première génération d'hybridation pour un tiers des individus mâles et se poursuivre en deuxième génération pour un autre tiers. L'analyse moléculaire des génomes cytoplasmiques des descendants de sexe mâle issus de croisements contrôlés impliquant un parent mâle

inconstant, en tant que parent femelle, et un mâle constant, en tant que parent mâle, permettrait de confirmer ou d'infirmer la possibilité d'un brassage entre chromosome sexuel et génome chloroplastique, et donc l'éventualité d'une incongruence entre les deux phylogénies. De plus, l'utilisation des descendants mâles de première génération dans des croisements ultérieurs et la ségrégation ou non du sexe dans les descendances ainsi obtenues devraient permettre d'inférer l'existence de supermâles YY.

Par contre, aucun cas de *femelle inconstante* (individu phénotypiquement femelle mais produisant parfois du pollen fertile) n'a été rapporté à ce jour (Testolin *et al.* 1995) et un seul cas d'hermaphrodisme a été décrit (McNeilage et Steinhagen 1998). Cette proportion apparemment plus grande de mâles inconstants est néanmoins à pondérer car, si la fertilité femelle se manifeste visuellement et simplement par la présence de fruits sur la plante, la fertilité mâle est beaucoup plus délicate à repérer.

Concernant le couplage entre chromosome X et génome mitochondrial, il n'est pas aussi strict qu'entre chromosome Y et génome chloroplastique puisque chacun des deux parents est supposé contribuer à hauteur d'un chromosome X chez les descendants femelles (sexe mâle hétérogamétique chez le kiwi). On ne s'attend donc pas à ce que les informations portées par le génome mitochondrial et celles que porte le chromosome X soient phylogénétiquement redondantes.

4

Reticulate evolution in kiwifruit (*Actinidia*, Actinidiaceae) deduced from the comparison of their maternal and paternal phylogenies

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article en préparation

Résumé

Les relations évolutives à l'intérieur d'*Actinidia*, genre connu pour le mode d'hérédité contrastée de ses plastes et de ses mitochondries, sont étudiées. L'analyse phylogénétique repose sur des données de sites de restrictions et de séquences du chloroplaste (cp) et de la mitochondrie (mt) : *mat*K, *psb*C-*trn*S, *rbc*L, *trn*L-*trn*F pour l'ADNcp; *nad*1-2/3 et *nad*4-1/2 pour l'ADNmt. La détection d'incongruences entre les jeux de données mitochondrial et chloroplastique donne un aperçu des relations phylogénétiques dans le genre, confirme certaines des réticulations déjà soulignées à partir de la phylogénie des gènes ribosomiques nucléaires et identifie de nouveaux événements d'hybridation/introgression. Un certain nombre d'événements d'hybridation/introgression sont documentés aux niveaux diploïdes et tétraploïdes. *Actinidia* a subi une évolution réticulée de grande échelle qui pourrait expliquer l'absence de discontinuités morphologiques claires entre les espèces.

Préambule

Comme nous l'avons montré précédemment, les deux génomes cytoplasmiques ont un mode d'hérédité distinct chez le kiwi. Dans ce contexte, identique à celui de la famille des Pinaceae (Wagner *et al.* 1987; Neale et Sederoff 1989; Wagner *et al.* 1991), l'exploitation des données moléculaires chloroplastiques et mitochondriales devient particulièrement intéressante puisque la lignée maternelle et la lignée paternelle peuvent être suivies en parallèle. On peut alors vérifier si les phylogénies chloroplastique (paternelle) et mitochondriale (maternelle) reflètent la même histoire évolutive, et en inférer l'histoire des espèces. Les similitudes comme les différences entre les deux topologies sont susceptibles de fournir des indications précieuses, permettant notamment la mise en évidence d'éventuelles hybridations. Ainsi, on distinguera un événement de polyploïdisation issu de l'addition de deux génomes complètement différenciés (*allopolyploïdie*) de celui issu d'un simple doublement d'un unique génome (*autopolyploïdie*). Un *néopolyploïde* est censé additionner les génomes nucléaires de ces ancêtres diploïdes. Pour les génomes cytoplasmiques, deux cas de figure peuvent se présenter. Lorsque chloroplastes et mitochondries sont transmis par la mère, cas le plus fréquent, un allopolyploïde résulte de l'addition de deux génomes nucléaires différenciés dans un cytoplasme purement maternel (Wendel 2000). Lorsque chloroplastes et mitochondries ont un mode de transmission uniparental et contrasté, non seulement le noyau mais également le cytoplasme témoignent du processus d'allopolyploïdisation, un parent fournissant le chloroplaste et l'autre la mitochondrie. En reconstruisant et en comparant l'arbre phylogénétique mitochondrial et l'arbre phylogénétique chloroplastique, on peut espérer distinguer l'autoploïdisation de l'alloploïdisation mais aussi identifier le ou les ancêtres du polyploïde. Par exemple, un néoautotétraploïde est censé former un groupe avec son unique ancêtre diploïde sur les deux arbres chloroplastique et mitochondrial alors qu'un néoallotétraploïde se trouvera placé à proximité de l'un de ses parents sur l'arbre chloroplastique et à côté de l'autre sur l'arbre mitochondrial (Figure 8). Non seulement l'identité des ancêtres est susceptible d'être révélée mais également le sens du croisement, une information généralement inaccessible à partir des phylogénies de gènes nucléaires.

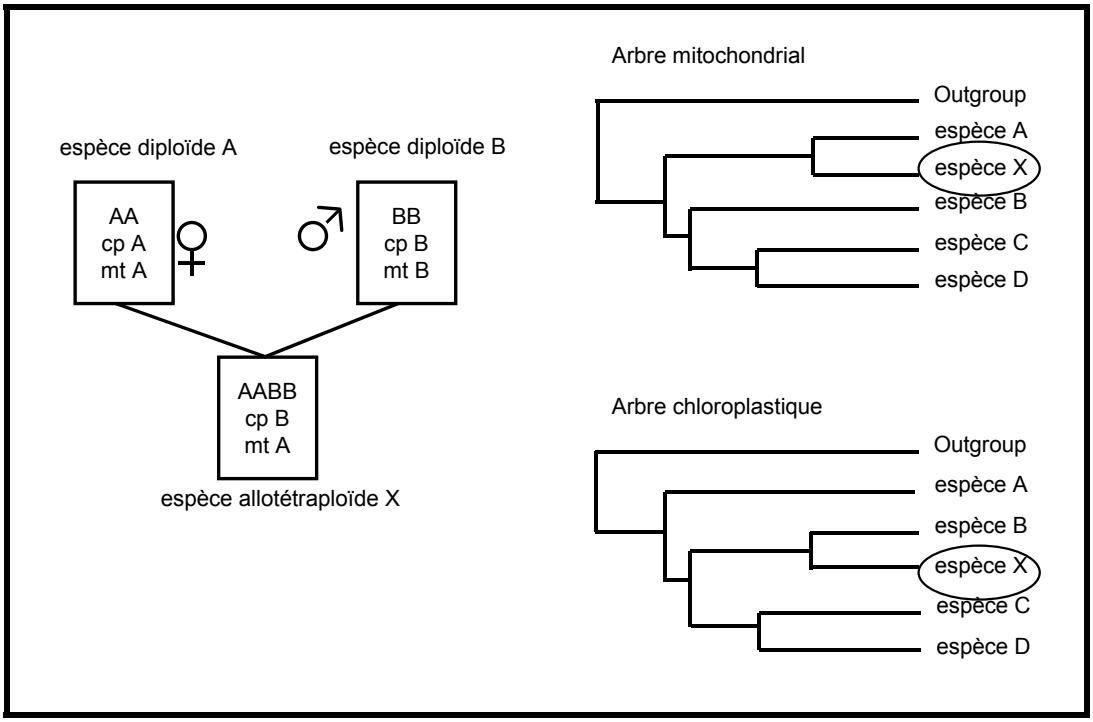

Figure 8 : Identification des ancêtres maternel et paternel d'une espèce allopolyploïde à partir des arbres phylogénétiques chloroplastique et mitochondrial chez *Actinidia*.

Fréquente et ancienne (Masterson 1994) chez les plantes supérieures, la polyploïdie est très présente dans le genre *Actinidia*. Les espèces actuelles de plantes qui possèdent un nombre de chromosomes élevé (typiquement supérieur à 12), ce qui est le cas du kiwi, sont d'ailleurs considérées par certains auteurs comme d'anciens polyploïdes (Stebbins 1950) dont l'évolution ultérieure a masqué l'origine. Parmi les angiospermes, on estime entre 2 % et 4 % les événements de spéciation impliquant une polyploïdisation (Otto et Whitton 2000). La production de gamètes non réduits est considérée aujourd'hui comme la voie de doublement chromosomique la plus fréquente dans la nature (Ramsey et Schemske 1998). Le genre *Actinidia* qui posséde un nombre chromosomique de base de 29 forme une série polyploïde qui se décline des diploïdes aux octoploïdes.

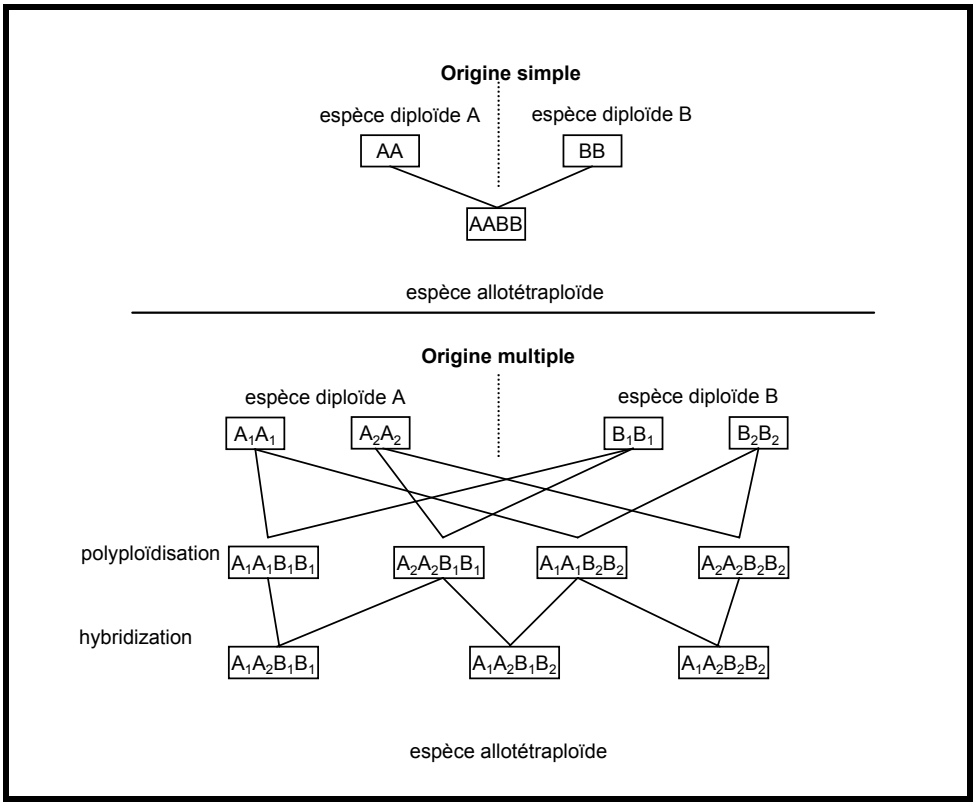

Figure 9 : Mode de formation des allopolyploïdes: vue traditionnelle (origine simple) et vue révisée (origine multiple) (d'après Soltis et Soltis 1999).

Les découvertes qui ont suivi les récentes avancées technologiques (marquage moléculaire, cartographie génétique, hybridation *in situ*, séquençage) ont profondément modifié notre compréhension du processus de polyploïdisation (Soltis et Soltis 1995; Petit *et al.* 1999; Soltis et Soltis 1999; Soltis et Soltis 2000) et notre connaissance des réorganisations intra- et intergénomiques (Rieseberg *et al.* 2000; Wendel 2000). Une des découvertes majeures concernant l'émergence des polyploïdes est leur *origine multiple* (Soltis et Soltis 1995) et consécutivement, leur importante diversité (Figure 9). Ceci a été vérifié expérimentalement à plusieurs reprises dans le cas des allopolyploïdes (p. ex. Doyle *et al.* 1990; Doyle *et al.* 1999), mais aussi, bien que plus rarement, dans le cas des autopolyploïdes (p. ex Segraves *et al.* 1999). De plus, l'isolement reproductif consécutif à la polyploïdisation est loin d'être total : l'existence de flux de gènes entre cytotypes est rapportée dans un nombre croissant de cas dans la littérature (revue dans Soltis et Soltis 1995; Petit *et al.* 1999). L'origine multiple des polyploïdes assortie du possible maintien d'un flux de gènes entre espèces diploïdes ancêtres et espèces polyploïdes contribue à donner aujourd'hui une image dynamique de l'évolution des polyploïdes qu'il nous appartenait de vérifier à l'échelle des *Actinidia*.

Title: Reticulate evolution in kiwifruit (*Actinidia,* Actinidiaceae) deduced from the comparison of their maternal and paternal phylogenies

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SUMMARY

Evolutionary relationships within *Actinidia*, a genus known for the contrasted mode of inheritance of its plastids and mitochondria, were studied. The phylogenetic analysis is based on chloroplast (cp) and mitochondrial (mt) restriction site and sequence data (*mat*K, *psb*C-*trn*S, *rbc*L and *trn*L-*trn*F for cpDNA; *nad*1-2/3 and *nad*4-1/2 for mtDNA). The detection of incongruences between the mtDNA and cpDNA data sets provides insights into the phylogenetic relationships within the genus, confirms some of the reticulations first emphasized from nuclear ribosomal gene phylogenies and diagnoses new hybridization/introgression events. A number of hybridization/introgression events at the diploid and tetraploid levels are documented. *Actinidia* has undergone extensive reticulate evolution, which could account for the lack of clear morphological discontinuities between species.

Key words: hybridization; mitochondrial indels; molecular phylogeny; *nad*1-2/3; *nad*4-1/2; *rbc*L; reticulate evolution; *trn*L-*trn*F;

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A plant cell typically hosts one nuclear and two organelle genomes (chloroplast and mitochondrial). The molecule of choice for molecular phylogenetic studies in the last decade has been the chloroplast genome. The nuclear genome has been relatively little used, in part due to the problem of distinguishing orthologous from paralogous genes (Olmstead and Palmer 1994). Similarly, the mitochondrial genome has been relatively neglected as a source of phylogenetic data for plants, due to its high degree of intramolecular recombination and its low rate of base substitution, except to investigate the relationships among phylogenetically distant lineages (Hiesel et al. 1994; Bowe et al. 2000; Chaw et al. 2000). This situation has changed in the last few years with a growing number of intrageneric or even intraspecific phylogenetic studies including information from the mitochondrial genome (e.g. Ronfort et al. 1995; Cho et al. 1998; Dumolin-Lapègue et al. 1998; Bakker et al. 2000; Freudenstein and Chase 2001; Anderberg et al. 2002).

In most plant species, both the chloroplast and the mitochondrial genomes are inherited from the mother. However, some plant species are known to transmit recurrently their mitochondrial and chloroplast genomes through opposite sexual partners. Such contrasted mode of organelle inheritance is found in several gymnosperms (Neale and Sederoff 1989) but also in some rare angiosperms, e.g. *Musa* (Fauré et al. 1994) and *Cucumis* (Havey et al. 1998). In such cases, the mitochondrial genome becomes particularly interesting for phylogenetic and population genetic purposes, since both maternal and paternal genetic lineages can be simultaneously traced (e.g. Wang et al. 2000). Investigating whether maternal, paternal and biparental (nuclear-based) phylogenies reflect the same evolutionary history, i.e. species history, is thus possible for these plant groups. Incongruences between a chloroplast gene tree and a mitochondrial gene tree will be especially informative in the context of a plant group subject to hybridization and polyploidization. They allow the distinction between allopolyploidy (merger of two fully differentiated nuclear genomes) and autopolyploidy (doubling of a single nuclear genome). When both organelles are maternally inherited as in most plant species, an allopolyploid speciation results in the merger of two differentiated nuclear genomes in the maternal cytoplasm (Wendel 2000). When organelle genomes show a contrasted and uniparental mode of inheritance, cytoplasmic genomes reflect the allopolyploidization process too, each parent species contributing one organelle genome.

Actinidia is an angiosperm plant genus well-documented for its paternal mode of chloroplast inheritance and its maternal mode of mitochondrial inheritance (Cipriani et al. 1995; Testolin and Cipriani 1997; Chat et al. 1999). The genus *Actinidia,* whose name reflects the peculiar radiating arrangement of the styles, consists of perennial, climbing or straggling, deciduous and dioecious plants. *Actinidia* belongs to the family Actinidiaceae together with the genera *Saurauia*

and *Clematoclethra* (Dickison 1972; Dickison et al. 1982). It occurs in Asia with a main center of diversity in southwestern China (Liang 1983). Some of the species, such as *A. polygama*, *A. arguta* and *A. chinensis*, have broad distributions, from Japan through northeastern Asia to western China (Li 1952), and exhibit geographical varieties. Most of them have more restricted ranges (Liang 1983). A subdivision of the genus *Actinidia* was first proposed by Dunn (1911). Following this first attempt, Li (1952), Liang (1983) and Cui (2002) successively proposed revisions (Table 1). Approximately 30 new species were described following botanical explorations undertaken during the twentieth century and added to the 24 species recognized by Dunn. Even during the past decade have additional taxa been described by Chinese botanists (e.g. Shi et al. 1994). Likewise, some taxa formerly considered as varieties have been later raised to species status (Liang and Ferguson 1986). Currently comprising some 50 species, *Actinidia* is divided into four infrageneric sections, namely *Leiocarpae*, *Maculatae*, *Stellatae* and *Strigosae*, on the basis of fruit (presence or absence of lenticels), pith (lamellate or nonlamellate) and hair (simple or stellate) characteristics. All the cytogenetical data reported until now suggest a basic chromosome number of $x = 29$ for the genus (Zhang and Beuzenberg 1983; McNeilage and Considine 1989). In sections *Leiocarpae* and *Stellatae*, tetraploid and hexaploid forms occur in addition to the diploid ones (Hopping 1994). Infraspecific variation in chromosome number appears to be common (Yan et al. 1997c). Previous molecular phylogenetic studies of infrageneric relationships in *Actinidia* based on chloroplast and nuclear data indicate that the current classification does not reflect the evolutionary history of the genus (Testolin and Ferguson 1997; Cipriani et al. 1998; Li et al. 2002). Furthermore, the systematic position of the family Actinidiaceae has long been unresolved, being included successively within Dilleniales, Theales or Ericales orders. Cladistic analyses based on morphological, anatomical and embryological features suggest that the family Actinidiaceae is basal within the Ericales (Judd and Kron 1993), a placement confirmed by chloroplast molecular data (Kron and Chase 1993; Anderberg et al. 2002).

TABLE 1. Comparison of the three main *Actinidia* taxonomic classifications.

Dunn (1911) 24 species	Li (1952) 36 species	Liang (1983) 53 species
Section Ampulliferae	**Section *Leiocarpae***	**Section *Leiocarpae***
		Serie Lamellatae
A. Giraldii	*A. arguta*	*A. arguta*
A. melanandra	*A. hypoleuca*	*A. globosa*
A. polygama	*A. kolomikta*	*A. henanensis*
A. rufa	*A. kwangsiensis*	*A. kolomikta*
A. tetramera	*A. maloides*	*A. maloides*
A. valvata	*A. melanandra*	*A. melanandra*
	A. polygama	*A. tetramera*
	A. purpurea	**Serie Solidae**
	A. tetramera	*A. macrosperma*
Section *Leiocarpae*	*A. valvata*	*A. polygama*
A. kolomikta		*A. valvata*
Section *Maculatae*	**Section *Maculatae***	**Section *Maculatae***
A. callosa	*A. asymmetrica*	*A. callosa*
A. coriacea	*A. callosa*	*A. chrysantha*
A. rubricaulis	*A. coriacea*	*A. cylindrica*
	A. fortunatii	*A. fasciculoides*
	A. glabra	*A. glauco-callosa*
	A. pilosula	*A. glaucophylla*
	A. rubricaulis	*A. gracilis*
	A. sabiaefolia	*A. indochinensis*
	A. trichogyna	*A. laevissima*
	A. venosa	*A. leptophylla*
		A. rubricaulis
		A. sabiaefolia
		A. trichogyna
		A. ulmifolia
		A. umbelloides
		A. venosa
Section *Vestitae*	**Section *Stellatae***	**Section *Stellatae***
		Serie Perfectae
A. Championi	*A. chinensis*	*A. chinensis*
A. chinensis	*A. eriantha*	*A. cinerascens*
A. Davidii	*A. fulvicoma*	*A. eriantha*
A. eriantha	*A. kiusiana*	*A. farinosa*
A. fulvicoma	*A. lanceolata*	*A. fulvicoma*
A. hemsleyana	*A. latifolia*	*A. lanceolata*
A. henryi	*A. longicauda*	*A. latifolia*
A. holotricha		*A. liangguangensis*
A. lanata		*A. rufotricha*
A. lanceolata		*A. styracifolia*
A. pachyphylla		*A. suberifolia*
A. rudis		*A. zhejiangensis*
A. strigosa		**Serie Imperfectae**
		A. grandiflora
		A. obovata
		A. pilosula
		A. sorbifolia
		A. stellato-pilosa

TABLE 1 (suite).

Dunn (1911) 24 species	Li (1952) 36 species	Liang (1983) 53 species
Not classified	**Section *Strigosae***	**Section *Strigosae***
A. Fortunati	*A. arisanensis*	*A. carnosifolia*
	A. hemsleyana	*A. chengkouensis*
	A. henryi	*A. fortunatii*
	A. holotricha	*A. hemsleyana*
	A. melliana	*A. henryi*
	A. petelotii	*A. holotricha*
	A. rubus	*A. melliana*
	A. rudis	*A. rubus*
	A. strigosa	*A. rudis*
		A. vitifolia

In order to understand the evolutionary history of the genus *Actinidia*, we compared the cpDNA and mtDNA phylogenies of most of the *Actinidia* species currently available in European and Chinese repositories. The reconstruction of the chloroplast genome history was mainly based on sequence data from two regions expected to evolve at contrasting rates: *rbc*L, a protein-coding gene, and the *trn*L-*trn*F region, which includes an intron and an intergenic spacer. For the mitochondrial genome, we examined variation in two mitochondrial introns, which are expected to exhibit more variation than the extremely conserved exons and which have been previously used to demonstrate the uniparental maternal mode of inheritance of the mitochondrial genome in *Actinidia* (Testolin and Cipriani 1997). The first one is located between exon2 and exon3 of subunit I of NADH dehydrogenase (*nad*1-2/3). The second is located between exon1 and exon2 of subunit IV of the same enzyme (*nad*4-1/2). Both regions were amplified by polymerase chain reaction (PCR) and subjected to restriction site analysis. By relying on both the maternal and the paternal lineages, we tried to elucidate the relationships within the *Actinidia* genus, to establish whether both the paternal and the maternal phylogeny fail to support the current morphologically-based classification, to track hybridization events and to infer the nature of the polyploidization processes that have affected this genus. In addition, we tried to clarify species boundaries within the three close relatives, namely *A. setosa* and the cultivated species *A. chinensis* and *A. deliciosa*.

MATERIALS AND METHODS

Taxon sampling-The taxa studied were selected from European and Chinese collections. Samples were obtained from European and Chinese collections. Vouchers of specimen coming from China and used in a previous published study have been deposited in HIB (Li et al. 2002). Additional vouchers for specimen coming from France have been collected and deposited in the Herbarium of the Musem National

d'Histoire Naturelle of Paris. A preliminary study involving a total of 82 accessions and representing 30 *Actinidia* species was conducted prior to sequencing to estimate within species polymorphism. Afterwards, a total of 42 accessions were chosen for *rbc*L and *trn*L-*trn*F sequencing (Table 2). Emphasis was placed on *Stellatae* section, especially *A. chinensis* and *A. deliciosa*, owing to their economic interests. *Saurauia* and *Clematoclethra*, the two other genera of Actinidiaceae, were used as outgroups.

DNA isolation, amplification and digestion-Total genomic DNA was isolated from leaf tissue according to a modified CTAB procedure (Chat et al. 1999). In order to evaluate inter and intraspecific variation, three cpDNA regions, i.e. an exon (*rbc*L), an intron (*mat*K) and an intergenic spacer (*psb*C-*trn*S), and two mtDNA introns (*nad*1-2/3 and *nad*4-1/2), were amplified using PCR and subsequently digested with 8 four-bases restriction enzymes used singly to generate restriction fragment length polymorphism (RFLP). Only losses and gains of restriction sites were scored for cpDNA.

DNA sequencing-One plant per haplotype was further sequenced to determine unambiguously the relative positions of both the restriction sites and the indels, except for *mat*K for which several *Actinidia* sequences had already been published. In addition, the 42 accessions selected were sequenced for both a coding and a non-coding cpDNA (*rbc*L, *trn*L intron and *trn*L-*trn*F intergenic spacer). The primers for PCR-amplification and for sequencing were as follows: *rbc*L (5' TTGGCAGCATTCCGAGTAA 3'; 5' TGTCCTAAAGTTCCTCCAC 3'), *mat*K (5' GTACTTGCTCATGATCATGG 3'; 5' CTAGCAAAGAAAGTCGAAG 3'), *psb*C and *trn*S (Demesure et al. 1995), *trn*L-*trn*F "c", "d", "e" and "f" (Taberlet et al. 1991), *nad*1-2/3 and *nad*4-1/2 (Demesure et al. 1995). PCR amplification conditions were as described in the references mentioned above. Double strand sequencing was performed directly from PCR amplification products. New additional primers were sometimes designed to achieve complete sequencing. Sequence contigs were assembled and completed sequences have been deposited in the EMBL database.

TABLE 2. Source and voucher specimen information for the plant material for which sequences were obtained. EMBL accession numbers for all *rbc*L and *trn*L-*trn*F sequences obtained and used in this study are also provided. EMBL number followed by an asterisk was taken from a previously published paper (Morton et al. 1997). Abbreviations used for collections: INRA: Institut National de la Recherche Agronomique-France, KBG: Kew Botanical Garden-Great Britain, LAM: Lycee Agricole de Montauban-France, UnUd: University of Udine-Italy, WIB: Wuhan Institut of Botany-China. Accession numbers from these collections follow the abbreviation (when available). (NA: not available)

Genus Section Species	Variety	Sex	2n	Source Repository, Accession	Voucher	EMBL accession number *rbc*L	 *trn*L-*trn*F
Actinidia Lindl							
Leiocarpae (Dunn) Li							
A. arguta (Sieb. et Zucc.) Planch. Ex Miq.	var. *arguta*	F	4x	WIB, 2-4-4AA981BJ	NA	AJ549049	AJ549004
A. kolomikta (Maxim. et Rupr.) Maxim		NA	2x	UnUd, P65[a]	NA	AJ549070	AJ549025
A. melanandra Franch.	var. *melanandra*	F	4x	WIB, 1-7-6ME981HB[b]	Li1059[b]	AJ549050	AJ549005
A. macrosperma C.F. Liang	var. *macrosperma*	M	4x	WIB, 2-1-1MA981HB	NA	AJ549053	AJ549008
	var. *mumoides*	M	4x	WIB, 2-9-5MB983JX[c]	NA	AJ549042	AJ548997
A. polygama (Sieb. et Zucc.) Maxim.		NA	2x	UnUd, P70	NA	AJ549071	AJ549026
A. rufa Planch. Ex Miq.		F	2x	WIB, 3-10-2RE990GX[b]	NA	AJ549059	AJ549014
A. valvata Dunn	var. *valvata*	F	4x	WIB, 2-12-1VA983GX[b]	Li1061[b]	AJ549052	AJ549007
Maculatae Dunn							
A. callosa Lindl.	var. *discolor*	NA	2x	WIB, 3-13-1CC984FG[b]	NA	AJ549065	AJ549020
	var. *henryi*	NA	2x	WIB, 3-8-1CF985GX[b]	NA	AJ549048	AJ549003
	var. *strigillosa*	M	4x	WIB, 3-14-2CG997GX[b]	Li1058[b]	AJ549061	AJ549016
A. chrysantha C.F. Liang		F	4x	WIB, 2-11-3CN998GX[b]	NA	AJ549035	AJ548990
A. cylindrica C.F. Liang	var. *cylindrica*	F	2x	WIB, 3-14-3CR990GX	NA	AJ549034	AJ548989
	var. *reticulata*	F	4x	WIB, 3-1-2CT983GX	NA	AJ549040	AJ548995
A. glaucophylla F. Chun	var. *glaucophylla*	F	2x	WIB, 2-6-4GB998GX	NA	AJ549043	AJ548998
	var. *rotunda*	F	2x	WIB, 3-14-4GE998GX[c]	NA	AJ549063	AJ549018
A. rubricaulis Dunn	var. *coriacea*	F	2x	WIB, 3-7-5RB983JX	NA	AJ549046	AJ549001
A. sabiaefolia Dunn		F	2x	WIB, 1-3-2SA998GX	NA	AJ549039	AJ548994
Strigosae Li							
A. hemsleyana Dunn	var. *hemsleyana*	F	2x	WIB, 1-6-4HA998GX[b,c]	NA	AJ549036	AJ548991
A. melliana Hand.-Mazz.		F	2x	WIB, 2-14MJ998GX	NA	AJ549067	AJ549022

[a] (Cipriani et al. 1998), [b] (Li et al. 2002), [c] (Huang et al. 2002a)

TABLE 2 (suite).

Genus Section Species	Variety	Sex	2n	Source Repository, Accession	Voucher	EMBL accession number *rbc*L	 *trn*L-*trn*F
Stellatae Li							
A. chinensis Planch.		F	2x	INRA, C8	Dumoulin 1	AJ549075	AJ549030
A. chinensis Planch.		F	4x	INRA, C9	Dumoulin 2	AJ549076	AJ549031
A. deliciosa	var. *deliciosa*	F	6x	INRA, 'Hayward'	Dumoulin 3	AJ549069	AJ549024
(A. Chev.) C.F. Liang et A.R. Ferguson	var. *chlorocarpa*	F	NA	WIB, 2-10-1DB990GX[c]	NA	AJ549041	AJ548996
A. eriantha Benth.	var. *alba*	F	NA	WIB, 2-9-3EB984FJ	NA	AJ549058	AJ549013
	var. *brunea*	F	NA	WIB, 3-14-5ED997GX	NA	AJ549062	AJ549017
	var. *calvescens*	F	2x	WIB, 2-13-5EC998GX[c]	NA	AJ549055	AJ549010
	var. *eriantha*	NA	2x	WIB, 3-11-3EA980JX[c]	NA	AJ549051	AJ549006
A. farinosa C.F. Liang		M	2x	WIB, 2-16-5FA997GX[c]	NA	AJ549064	AJ549019
A. fulvicoma Hance	var. *fulvicoma*	M	2x	WIB, 3-12-3FF982JX	NA	AJ549066	AJ549021
	var. *lanata*	M	2x	WIB, 2-12-2FG998GX[b,c]	NA	AJ549057	AJ549012
A. grandiflora C.F. Liang		F	4x	WIB, 2-6-2GH990GX	NA	AJ549044	AJ548999
A. guilinensis C.F. Liang		F	2x	WIB, 3-3-2GI990GX	NA	AJ549047	AJ549002
A. hubeiensis C.F. Liang		F	2x	WIB, 2-13-5HU998GX	NA	AJ549056	AJ549011
A. lanceolata Dunn			2x	UnUd, P83[a]	NA	AJ549072	AJ549027
A. latifolia (Gardn et Champ.) Merr.	var. *latifolia*	F	2x	WIB, 3-2-1LC984HN	NA	AJ549037	AJ548992
A. lijiangensis C.F. Liang et Y.X. Lu		F	2x	WIB, 3-6-2LG990GX	NA	AJ549045	AJ549000
A. persicina C.F. Liang		F	2x	WIB, 3-4-2PS984FJ[c]	NA	AJ549068	AJ549023
A. setosa (Li) C.F. Liang et A.R. Ferguson		F	2x	LAM, ST2	Dumoulin 4	AJ549073	AJ549028
A. styracifolia C.F. Liang		F	2x	WIB, 3-5-1SF990GX[b,c]	NA	AJ549033	AJ548988
A. zhejiangensis C.F. Liang		NA	2x	WIB, 2-8-2ZA992ZJ[b]	NA	AJ549038	AJ548993
Clematoclethra							
C. lasioclada Maxim.		NA	NA	KBG	Edinburg 1909-1001 K	Z80172*	AJ549032
Saurauia							
S. nepaulensis DC.		NA	NA	KBG	M.W. Chase 1096 K	AJ549074	AJ549029

[a] (Cipriani et al. 1998), [b] (Li et al. 2002), [c] (Huang et al. 2002a)

Phylogenetic analyses

Chloroplast data-Multiple alignments of the sequences were obtained using the ClustalX program (Thompson et al. 1997). Insertions/deletions (indels) in the *trn*L-*trn*F alignment were coded using the method of Simmons and Ochoterena (Simmons and Ochoterena 2000) implemented in the program GapCoder (**http://www.trinity.edu/nyoung/GapCoder)**, and appended to the *trn*L-*trn*F sequence matrix. Phylogenetic analyses were all performed using PAUP* 4.0b10 (Swofford 1998). The three chloroplast data sets, i.e. restriction sites (*rbc*L, *mat*K and *psb*C-*trn*S), *rbc*L sequences and *trn*L-*trn*F sequences (with gaps either coded or ignored) were analyzed separately or combined except the *rbc*L restriction sites that were discarded to avoid redundancy with *rbc*L sequences. Parsimony analyses used heuristic searches with 1000 random addition replicates, tree bisection-reconnection (TBR), branch swapping, MulTrees on, with all character states unordered and equally weighted and gaps coded as previously described. The congruence of the *rbc*L and *trn*L-*trn*F sets was assessed using incongruence length difference (Farris et al. 1995) calculated using the partition homogeneity test in PAUP*. This test was performed with 100 replications of heuristic search. Strict and majority rule consensus trees were calculated from all most parsimonious trees. The robustness of nodes was inferred by a bootstrap analysis of 1000 replicates of heuristic search on the combined data set and by calculating decay values (Bremer 1994) with Autodecay 4.0 software (Eriksson 1999). In order to explore alternative hypotheses against the data, additional heuristic searches were performed using the option "enforce topological constraints". The g_1 statistic was calculated using all the characters on a subset of 12 *Actinidia* taxa representing the major clades of the strict consensus tree. Characters of the combined data set were also successively weighted (Farris 1969) based on the rescaled consistency index (RC), a base weight of 1000, and their maximum value if more than one tree was found. Subsequently, a heuristic search was performed with the same options as previously described except for character weight. Successive rounds of weighting/searching were performed until in two successive rounds the same tree length was obtained. In order to explore more fully the sister-species relationships within *Leiocarpae*, a parsimony analysis by unweighted exhaustive search was also performed using the same options as above.

Mitochondrial data-Multiple alignments of the sequences of each mitotype were performed as for chloroplast sequences. To reconstruct phylogenetic relationships, it is preferable to choose the method that requires the least assumptions of state changes in order to avoid misinterpretation. When coding length variation, we therefore used simultaneously two of the methods described in Freudenstein and Chase (2001) when reconstructing mitochondrial phylogeny of Orchidaceae. A single multistate unordered character (each different gap string coded as a state of the character) was used for *nad*1-2/3

where both nested and overlapping gap strings were found, whereas binary characters (presence/absence of a specific gap) were preferred for *nad*4-1/2. A parsimony exhaustive search was then performed on the mitotype matrix containing gap information.

RESULTS

Chloroplast data

Restriction sites-A total of 26 variable restriction sites were revealed among the 82 accessions (81 from *Actinidia* and one from *Clematoclethra*), respectively four for *rbc*L (1118 bp), 15 for *mat*K (1221-1227 bp) and seven for *psb*C-*trn*S (1604-1615 bp). Among them, 10 character states were autapomorphies (Table 4), three for *Clematoclethra* and seven for *Actinidia*. Of the 21 *Actinidia* species represented by at least two accessions, eight species showed intra-specific restriction site variation (Table 4). In contrast, 13 species including up to four accessions did not exhibit any site polymorphism. The level of variation displayed by restriction site analysis of the *rbc*L region suggested that this region was likely to be phylogenetically informative and nearly complete sequencing was performed. In order to get the full information from this region, sequencing was therefore undergone. When several accessions were available within a taxon (species or variety), only one accession representing the most common restriction profile for that taxon was sequenced (listed in Table 2).

TABLE 3. Restriction site mutations (PCR-RFLP) scored for phylogenetic analysis of cpDNA in *Actinidia* genus (81 accessions) and outgroup *Clematoclethra*.

Character No.	Code	CpDNA region, restriction enzyme	Polymorphism (fragment sizes in kb)[1]
1	r1	*rbc*L, *Alu* I	771→132+639
2	r2	*rbc*L, *Alu* I	283→189+94
3	r3	*rbc*L, *Tru1* I	413→376+37
4	r4	*rbc*L, *Tru1* I	287→156+131
5	m1	*matK*, *Hinf* I	423→(309)+(114)
6	m2	*mat*K, *Hinf* I	220→50+170
7	m3	*mat*K, *Nde* II	261→77+184
8	m4	*mat*K, *Nde* II	417→110+307
9	m5	*mat*K, *Rsa* I	1221→939+282
10	m6	*mat*K, *Taq* I	668→31+631
11	m7	*mat*K, *Taq* I	295→63+232
12	m8	*mat*K, *Taq* I	295→143+152
13	m9	*mat*K, *Taq* I	631→465+166
14	m10	*mat*K, *Taq* I	465→111+354
15	m11	*mat*K, *Tru1* I	150→30+120
16	m12	*mat*K, *Tru1* I	246→150+96
17	m13	*mat*K, *Tru1* I	336→174+162
18	m14	*mat*K, *Tru1* I	450→336+114
19	m15	*mat*K, *Tru1* I	450→379+71
20	cs1	*psb*C-*trn*S, *Alu* I	648→552+96
21	cs2	*psb*C-*trn*S, *Hha* I	887→(131)+(730)
22	cs3	*psb*C-*trn*S, *Hha* I	887→555+314
23	cs4	*psb*C-*trn*S, *Msp* I	1042→432+610
24	cs5	*psb*C-*trn*S, *Msp* I	381→270+111
25	cs6	*psb*C-*trn*S, *Nde* II	255→98+157
26	cs7	*psb*C-*trn*S, *Rsa* I	1337→1276+61

[1] The exact sizes of the restriction fragments were deduced from the sequences except when indicated in parentheses.

TABLE 4. Character matrix of the restriction site mutations (0/1: absence/presence of restriction site). The number of accessions studied is indicated in parentheses after each species name. The number of accessions having or not the restriction site is indicated in superscript in the table. Column titles refer to the restriction site code attributed in Table 3. Autapomorphies underlined.

Taxa	r1	r2	r3	r4	m1	m2	m3	m4	m5	m6	m7	m8	m9	m10	m11	m12	m13	m14	m15	c1	c2	c3	c4	c5	c6	c7
Clematoclethra	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?
A. arguta (5)	0	0	0	$0^2/1^3$	0	1	1	0	0	1	1	1	0	0	0	0	0	0	0	1	0	$0^2/1^3$	0	0	0	0
A. callosa (4)	1	0	0	1	0	0	1	0	0	1	$0^2/1^2$	1	0	0	0	$0^2/1^2$	0	0	0	1	0	0	$0^1/1^3$	0	0	$0^2/1^2$
A. chinensis (11)	1	0	0	1	0	0	1	$0^1/1^{10}$	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1
A. chrysantha (2)	1	0	0	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1
A. cylindrica (3)	1	0	0	$0^2/1^1$	0	0	1	0	0	1	1	1	$0^1/1^2$	$0^1/1^2$	0	0	0	0	0	1	0	0	0	0	0	$0^1/1^2$
A. deliciosa (7)	1	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	$0^6/1^1$	0	1
A. eriantha (4)	1	0	0	$0^3/1^1$	0	0	1	$0^1/1^3$	0	1	$0^1/1^3$	1	$0^1/1^3$	$0^1/1^3$	0	0	0	0	0	1	0	0	0	0	0	1
A. farinosa (1)	1	0	0	1	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A. fulvicoma (3)	1	0	0	1	0	0	1	0	0	1	1	1	$0^2/1^1$	0	0	0	0	0	0	1	0	0	0	0	0	0
A. glaucophyla (2)	1	0	0	1	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	$1^1/0^1$	0	0	0	0	$0^1/1^1$	0
A. grandiflora (1)	1	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1
A. guilinensis (1)	1	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1
A. hemsleyana (2)	1	0	0	1	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1
A. hubeiensis (1)	1	0	0	1	0	0	1	0	0	1	1	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0
A. kolomitka (2)	1	0	0	1	0	0	1	0	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1
A. lanceolata (1)	1	0	0	1	0	0	1	0	0	1	1	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0
A. latifolia (3)	1	0	0	0	0	0	1	0	0	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	1
A. lijiangensis (1)	1	0	0	1	0	0	1	0	0	1	1	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0
A. macrosperma (4)	1	0	0	1	0	0	1	0	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1
A. melanandra (3)	0	0	0	1	0	1	1	0	0	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0
A. melliana (1)	1	0	0	1	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0
A. persicina (2)	1	0	0	1	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1
A. polygama (2)	1	0	1	1	0	0	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1
A. rubricaulis (3)	1	0	0	1	0	0	1	0	0	1	1	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0
A. rufa (3)	1	0	0	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1
A. sabiaefolia (3)	1	0	0	1	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	1	**1**	0	0	0	0	1
A. setosa (2)	1	0	0	1	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1
A. styracifolia (1)	1	0	0	0	0	0	1	0	0	1	1	1	1	1	0	0	0	0	0	1	0	0	0	0	0	1
A. valvata (2)	1	0	1	1	0	0	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1
A. zhejiangensis (1)	1	0	0	1	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1

Sequences-The region of the *rbc*L gene analyzed here comprised 1000 characters (corresponding to base positions 174-1173 of the *rbc*L sequence of *Nicotiana tabacum* L.). Among the 49 variable sites, 32 (65%) were phylogenetically informative (Table 5). Parsimony analysis of the *rbc*L matrix yielded eleven equally parsimonious trees of 65 steps, consistency index (CI) excluding uninformative sites = 0.79 and retention indice (RI) = 0.91. Lengths of the *trn*L-*trn*F sequences prior to alignment varied from 913 to 931, 957 being the final length of the alignment. The amplicon, which includes the *trn*L intron and *trn*L-*trn*F intergenic spacer, displayed simple sequence repeats (SSR) and several indels of 4-8 bases, representing additional variable characters. The SSRs, prone to homoplasy (Doyle et al. 1998), were subsequently excluded from the phylogenetic analyses whereas the indels, when parsimony-informative, were treated as binary characters and appended to the *trn*L-*trn*F sequence matrix. The number of substitutions in the *trn*L-*trn*F alignment was 84 of which 37 (44%) were informative. The *trn*L-*trn*F parsimony analyses produced five equally parsimonious trees of 107 to 96 steps depending on whether gaps were included or not. Including gap codes in the *trn*L-*trn*F sequence matrix did not modify the topology of the strict consensus tree (data not shown) and increased slightly the RI (Table 5). The partition-homogeneity test indicated that the two sequence matrices were not statistically incongruent (P = 0.07). In spite of a lack of resolution in the *rbc*L tree, yielding several polytomies, the majority rule consensus tree of *trn*L-*trn*F is topologically similar with that of *rbc*L with respect to the major clades. For example, in both trees, the *Leiocarpae* section is paraphyletic and all the seven *Leiocarpae* species but one (*A. rufa*) are sister groups to the rest of the genus. The only substantial incongruence between the two topologies is the placement of *A. rufa*.

TABLE 5. Comparison of lengths and indices for the analyses of separate and combined chloroplast data sets. CI and RI are the consistency and retention indices, respectively.

Data set	Number of			Tree length	CI	RI
	variable characters (%)	parsimony informative characters	most parsimonious trees			
*rbc*L	49 (5%)	32	11	65	0.79	0.91
*trn*L-*trn*F (gaps treated as missing data)	84 (8.8%)	37	5	96	0.90	0.93
*trn*L-*trn*F (informative gaps coded[1])	95 (9.9%)	41	5	107	0.91	0.94
*mat*K and *psb*C-*trn*S restriction sites	21	13	6676	24	0.88	0.94
combined	165	86	4	202	0.84	0.91

[1] SSR loci excluded

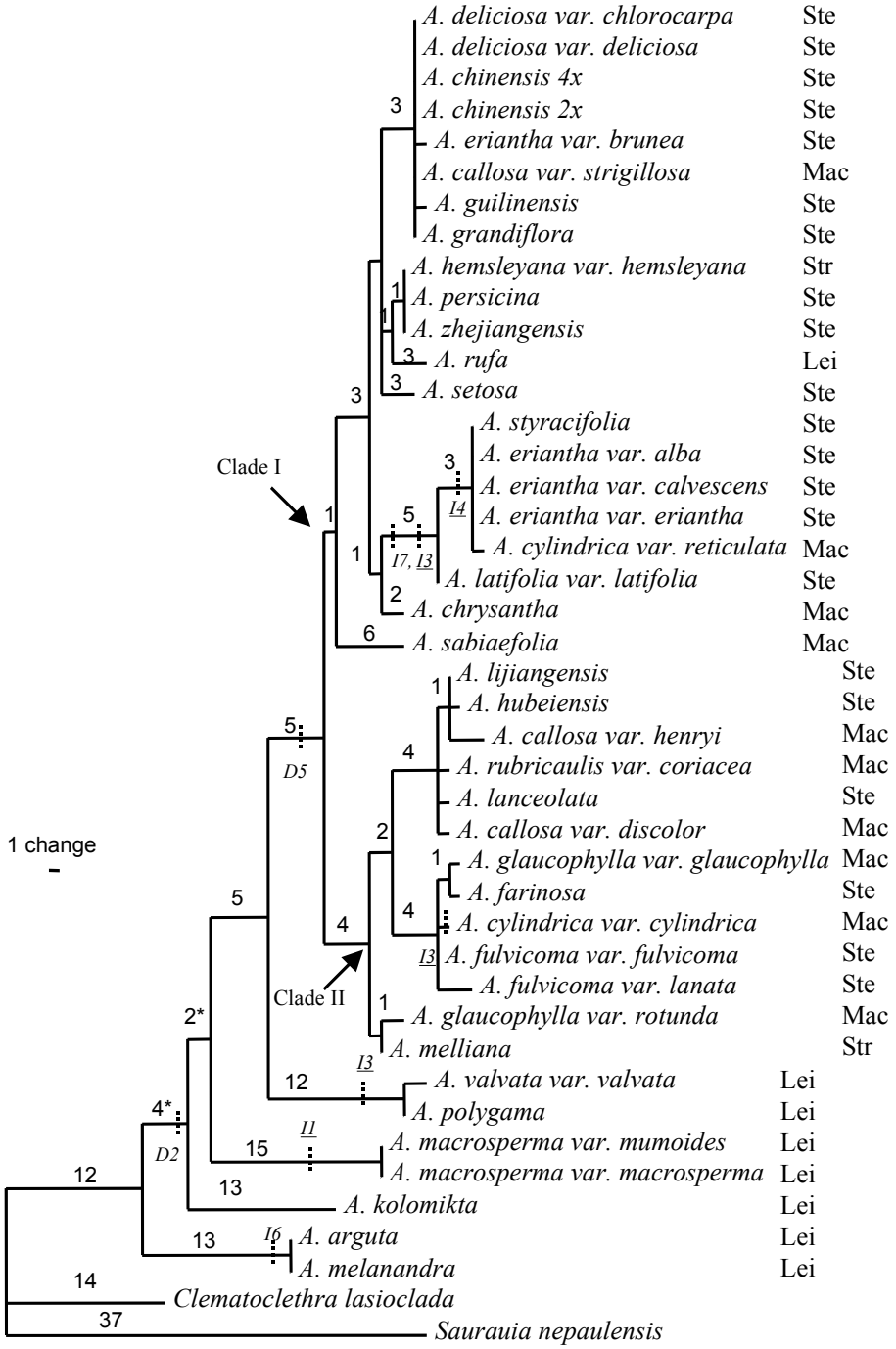

Fig. 1. One of four most-parsimonious trees resulting from a heuristic search of 41 sequences (*rbc*L and *trn*L-*trn*F) and restriction sites (*mat*K and *psb*C-*trn*S) of 30 *Actinidia* species. The two other recognized genus of the Actinidiaceae, namely *Clematoclethra* and *Saurauia*, served as outgroups. Length = 202 steps, CI = 0.84, RI = 0.91. Branch lengths are proportional to the number of changes. Transitions in the seven informative indels of *trn*L-*trn*F sequences (ID1-7) are indicated on the phylogram by dotted bars. In the phylogenetic reconstruction, ID1, ID3 and ID4 (SSRs) were treated as missing (underlined on the phylogram), ID2 and ID5-7 (indels) were coded as additional characters. The polarity inferred were as follows (I for insertion, D for deletion): D2 is a deletion of 8 bp, D5 is a 4 bp deletion and I6 and I7 represent each a 5 bp insertion. Asterisks indicate branches collapsing in the strict consensus. The sections of the classification of Liang (1983) **are listed (Lei: *Leiocarpae*, Mac: *Maculatae*, Ste: *Stellatae*, Str: *Strigosae*).**

The combined data matrix had 1990 characters (1957 from sequences, 11 from *trn*L-*trn*F gaps and 22 from restriction sites), of which 165 characters are variable and 86 potentially phylogenetically informative. As suggested by the shape of tree length distribution (g1 statistic = -0.88), there is phylogenetic signal in the data. Parsimony analysis of this combined data matrix resulted in one island of four equally parsimonious trees of 202 steps (CI = 0.84, RI = 0.91), which have the same topology except for the placement of *A. arguta*, *A. melanandra* and *A. kolomikta*. One of them is shown in Fig. 1. The state changes in indels and SSRs in the *trn*L-*trn*F alignment are indicated on the tree. As expected, one of the three *trn*L-*trn*F phylogenetically informative SSRs (ID3) appears to be highly homoplasic with the same state evolving three times independently. In contrast, the other type of insertion/deletions mapped unambiguously as synapomorphies with only one state change. This tree (Fig. 1) as well as the strict consensus tree (Fig. 2) showed two major clades. Most of the accessions (15 out of 20) included in clade I are from the *Stellatae* section whereas clade II, more strongly supported, includes a well-balanced number of *Maculatae* and *Stellatae* accessions. Section *Stellatae* and *Maculatae* appear therefore polyphyletic. *A. hemsleyana*, one of the only two *Strigosae* species examined in this study, is embedded within clade I, whereas the other one, *A. melliana*, falls into clade II. Concerning *Leiocarpae*, one species, *A. rufa* is placed in clade I, far from the other seven *Leiocarpae* species. *Leiocarpae* forms a paraphyletic group with four clades at the base of the tree, although it is the most distinguishable section in terms of morphological characters. Successive weightings produced three trees differing only by the placement of *A. kolomikta*. The combination of these three trees as a strict consensus tree is identical in topology to the strict consensus tree of the unweighted analysis shown in Fig. 2. The unweighted exhaustive parsimony analysis focusing on *Leiocarpae* yielded four most parsimonious trees (151 steps long, CI = 0.88, RI = 0.82). Section *Leiocarpae* appears paraphyletic in all these four trees (strict consensus tree in Fig. 3).

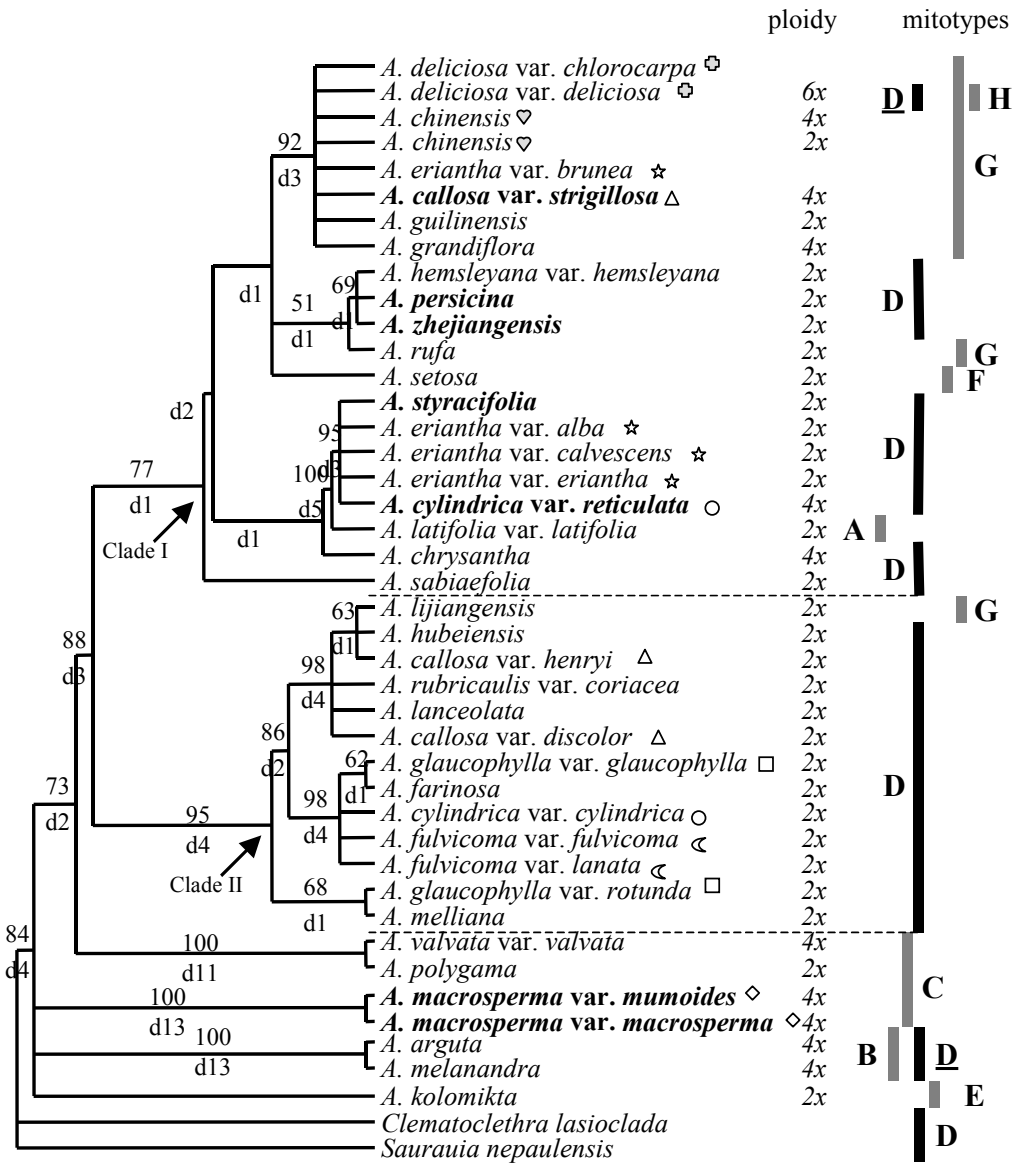

Fig. 2. Strict consensus of the four equally parsimonious trees of 41 taxa from 30 *Actinidia* species based on chloroplast (*rbc*L and *trn*L-*trn*F) sequences and restrictions site (*mat*K and *psb*C-*trn*S) data. Numbers below branches refer to decay index and numbers above branches indicate bootstrap proportions (>50%) from 1000 replicates. Both ploidy and mtDNA haplotype (underlined in case of intraspecific polymorphism) which occurred within each sequenced individual are listed after species and variety names. Black and shaded bars indicate plesiomorphic and apomorphic mitotypes, respectively. Symbols are used to label several taxa of the same taxonomic species. Taxa with a probable reticulate origin as deduced from the presence of distantly related ITS sequences (Li et al. 2002) **are indicated in boldface.**

TABLE 6. Description of the eight distinct mitotypes found in this study.

Mitotype	*nad*1-2/3			*nad*4-1/2				
	785-806 [1]	Coding	EMBL accessions No.	801-806 [1]	1068-1073 [1]	1338-1344 [1]	Coding	EMBL accessions No.
A	----------	0	NA	------	------	--------	0 0 0	AJ536479
B	----------	0	NA	TTCAAA	------	TGTTCGC	1 0 1	AJ536481
C	----------	0	NA	------	CTAGAT	TGTTCGC	0 1 1	AJ536478
D	----------	0	AJ536470; AJ536476	------	------	TGTTCGC	0 0 1	AJ536480; AJ536483
E	CTTTTT-ATTTT----------	1	AJ536469	------	------	TGTTCGC	0 0 1	NA
F	CTTTTATATTTT----------	2	AJ536467; AJ536468	------	------	TGTTCGC	0 0 1	NA
G	-------ATTTTATTTT-----	3	AJ536475; AJ536471; AJ536472; AJ536473; AJ536474	------	------	TGTTCGC	0 0 1	AJ536482
H	-------ATTTTATTTTATTTT	4	AJ536477	------	------	TGTTCGC	0 0 1	NA

[1] Position of the indels in the aligned sequences.

Mitochondrial restriction sites-Mutational loss or gain of restriction sites were found neither in *nad*1-2/3 nor in *nad*4-1/2. However, seven indels were found among the 81 accessions. Partial sequencing and alignment further helped to identify each of the eight mitotypes formerly observed on restriction profiles by the exact length, base composition and position of each insertion/deletion events (Table 6). However, partial sequencing failed to reveal additional parsimony-informative nucleotide substitutions. The three indels observed within *nad*4-1/2 are distant by 200-300 bases whereas all the four indels found within *nad*1-2/3 are located at the same median position in the amplicon. An equally weighted parsimony analysis conducted on the mitotype matrix yielded 37 equally parsimonious trees. Due to the small amount of phylogenetic information, the strict consensus tree resulted in a large polytomy linking all the eight mitotypes (data not shown). As a consequence, information relative to the mitochondrial genome (mitotype) and the nuclear genome (ploidy) were mapped onto the chloroplast-based strict consensus tree. Among the eight mitotypes identified, mitotype C, all three mitotypes A, E and F and mitotype H appear to be series-, species- and clone-specific, respectively (Figure 2). Furthermore, three mitotypes (B, C and E) were typical of the *Leiocarpae* section. Mitotype G is found mainly in *A. chinensis* and *A. deliciosa*, as well as in some close relatives. Mitochondrial intraspecific polymorphism was detected in five species, i.e. *A. arguta* (B + D), *A. callosa* (D + G), *A. deliciosa* (D + G + H), *A. eriantha* (D + G) and *A. melanandra* (B + D). Mitotype B is present in *A. arguta* and *A. melanandra* but absent from the two plants selected to represent those taxa on the chloroplast phylogenetic trees.

DISCUSSION

***Maternal phylogeny*-**This study represents the first use of molecular markers of mitochondrial origin to characterize genetic diversity in *Actinidia*. A first conclusion is the occurrence of mtDNA diversity among the *Actinidia* species. Both mitochondrial regions chosen for this study displayed variations among and sometimes within *Actinidia* species. However, only informative indels were revealed. Present in the two outgroups, mitotype D appears to be ancestral. Clearly, one five-state character (*nad*1-2/3) and three binary characters (*nad*4-1/2) are far too few to properly resolve the maternal phylogeny of 30 *Actinidia* species and additional molecular data are needed to provide more resolution.

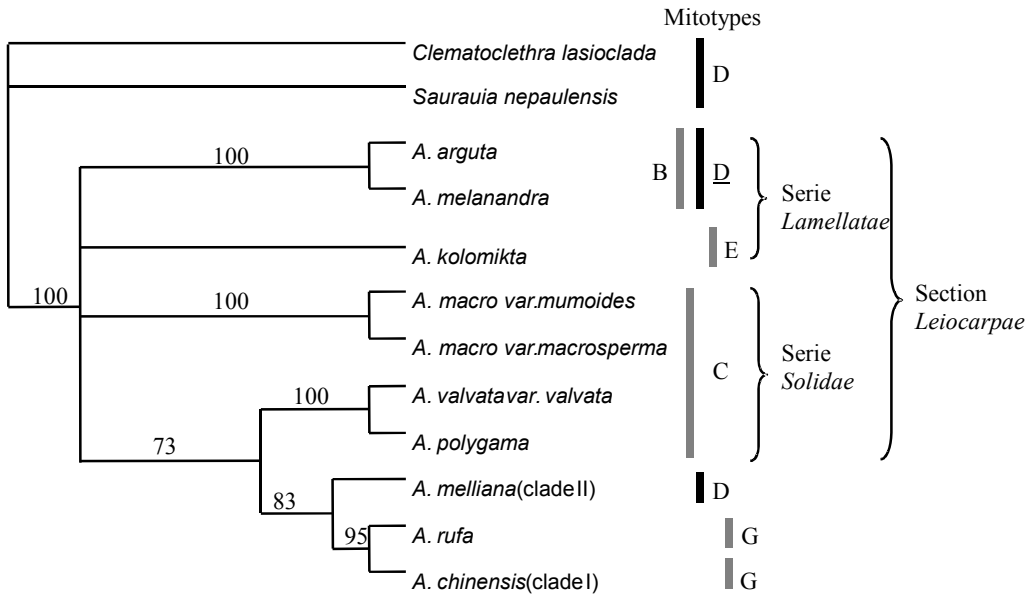

Fig. 3. Strict consensus of the four equally parsimonious trees obtained following an exhaustive search. Length = 151 steps, CI = 0.88, RI = 0.82. The sudivisions of the classification of Liang (1983) **are listed. Section *Leiocarpae* appears paraphyletic. Black and shaded bars indicate plesiomorphic and apomorphic mitotypes, respectively.**

Congruences between maternal and paternal phylogenies- Even if they provide little resolution on their own, mapping the mitotypes onto the paternal chloroplast-based phylogeny clarifies part of the phylogenetic history of the *Actinidia* species. In several cases indeed, the relationships deduced from the mitochondrial characters do not conflict with those revealed in the chloroplast-based parsimony analysis, as the plants sharing the same derived mitotypes are also closely related in the paternal tree. Two mitotypes are shared by sister taxa on the cpDNA tree (B by *A. arguta* and *A. melanandra*, C by A. *valvata* and *A. polygama* and by var. *mumoides* and var. *macrosperma* of *A. macrosperma*), and mitotype G is shared by most members of the large polytomy which includes the cultivated species. This result is not so trivial, since many *Actinidia* species can be crossed under experimental conditions (Hirsch et al. 2001), suggesting that interspecific hybridization could have played a major role in the evolutionary history of the genus.

Incongruences between maternal and paternal phylogenies- Despite this overall pattern, our data also provide evidence for three striking incongruences between maternal and paternal phylogenies. These incongruences all involve mitotype G: its unexpected presence in *A. rufa* and *A. lijiangensis* and its absence in one individual of *A. deliciosa*. The presence of mitotype G in *A. lijiangensis* could result

from homoploid hybrid speciation or from introgression. Unfortunately, this species was not included in the study conducted by Li et al. (2002) with nuclear ribosomal internal transcribed spacer (ITS) sequences, so its putative hybrid origin cannot be assessed. The apparent conflicts for the other two species, i.e. *A. rufa* and *A. deliciosa*, could be due to ancestral polymorphism, resulting in incomplete lineage sorting in the case of *A. deliciosa*. The absence or the low levels of intraspecific polymorphism displayed by the *A. chinensis*, *A. deliciosa* and *A. rufa* ITS nuclear sequences (Li et al. 2002) suggest that these three species are not of hybrid origin, therefore supporting the hypothesis of an ancestral polymorphism. Furthermore, *A. rufa* belongs to a clade, which emerges as sister to the largely unresolved group including *A. chinensis* and *A. deliciosa* in the cpDNA tree. However, to attribute the presence of mitotype D and G within *A. deliciosa* to incomplete lineage sorting requires that *A. deliciosa* inherited that polymorphism from its progenitors. The view supported by Testolin and Ferguson (Testolin and Ferguson 1997) on the basis of isozyme evidence is that *A. deliciosa* have originated from the sole *A. chinensi*s, and the nuclear ITS tree (Li et al. 2002) appears too to favor a hypothesized autopolyploid origin of *A. deliciosa* from *A. chinensis*.. Nevertheless, mitotype D has been found neither in the diploid cytotypes nor in the tetraploid cytotypes of *A. chinensis* sampled in the present study. If the absence of mitotype D in *A. chinensis* is confirmed, the unique mitotype D found in *A. deliciosa* would better originate from a hybridization/introgression event involving one particular plant or population. Increasing sampling, particularly within *A. chinensis* and outside the geographical area previously prospected, and sequencing the nuclear ITS sequences of that particular plant are thus needed to provide conclusive arguments.

Intraspecific polymorphism as an indicator of reticulation events-Four of the eight species that were represented by at least two taxa, i.e. *A. callosa*, *A. cylindrica*, *A. eriantha* and *A. glaucophylla*, showed intraspecific polymorphism. Two of them, *A. glaucophylla* and *A. cylindrica*, showed within species variation in cpDNA only whereas *A. eriantha* and *A. callosa* revealed intraspecific variation in both cpDNA and mtDNA. Although the distinct varieties of *A. callosa*, *A. cylindrica*, *A. eriantha* and *A. glaucophylla* have not been raised to specific status, they have distinct evolutionary histories. The most plausible scenario is that some of the varieties have experienced reticulations whereas others have not. Nuclear data are in agreement with this hypothesis. For instance, the tetraploid *A. callosa* var. *strigillosa* combines two types of ITS nuclear sequences, both "*chinensis*-like" and "*callosa*-like", which provide convincing evidence of a hybrid origin. This contrasts with the low divergence of the nuclear ITS sequences of the other two diploid varieties of that species (*henryi* and *discolor*) (Li et al. 2002). In other respects, the tetraploid *A. cylindrica* var. *reticulata* (clade I on the chloroplast-

based tree) appears to be distantly related to the conspecific diploid var. *cylindrica* (clade II) but closely related to *A. eriantha*, strongly suggesting that it is an allopolyploid arising by hybridization with a male parent similar to the present-day *A. eriantha*. One of the two *A. glaucophylla* varieties may also be derived from hybridization, although this is not so clear due to less divergent chloroplast sequences. Finally, because *A. eriantha* var. *brunea* and *A. callosa* var. *strigillosa* combine both distantly related chloroplast sequences and distinct mitotypes compared to the other diploid conspecific taxa, they must have undergone more than a single hybridization event.

Relationships between A. setosa, A. chinensis and A. deliciosa-The island species *A. setosa*, endemic of Taiwan, was first considered by Li (1952) to be conspecific with the mainland *A. chinensis* found in China. *A. setosa* has been rarely included in the morphological and molecular studies and its phylogenetic relationships with *A. chinensis* and *A. deliciosa* remain unclear. However, in accordance with the view of Liang and Ferguson (1985), we think that *A. setosa* can be treated as a separate taxon due to its molecular divergence from *A. chinensis* and *A. deliciosa* as judged from both its cpDNA and mtDNA. In contrast, *A. chinensis* and *A. deliciosa* have been previously recognized as a species complex (Testolin et al. 1997; Cipriani et al. 1998). Since then, more evidence has been provided for such a hypothesis. As shown by artificial hybridizations, fertility between *A. chinensis* diploids and *A. chinensis* tetraploids (Guijun et al. 1994) as well as between *A. chinensis* tetraploids and *A. deliciosa* hexaploids (Hirsch et al. 2001) is reduced but sufficient to maintain current gene flow between all three cytotypes. Likewise, since there are large zones of contact between *A. chinensis* and *A. deliciosa* (Ferguson 1990a), gene exchanges are expected to occur in nature too. The existence of transitional forms in the areas where the two species overlap suggests that hybridization occurs (Ferguson 1990a). In line with this finding, the group consisting of *A. chinensis* and *A. deliciosa* is monophyletic relative to the other *Actinidia* species for the chloroplast data (present study and Li et al. 2002) and for the nuclear data (Li et al. 2002). Recent divergence and high levels of species interfertility thus provide considerable support for such a species complex hypothesis.

Systematic implications-The monophyly of the *Actinidia* genus demonstrated using both *rbc*L and *trn*L-*trn*F sequences and one member of each of the two other genera of the Actinidiaceae as outgroups is well supported by the decay index (4), the number of synapomorphies (11) as well as the bootstrap value (85%). On the other hand, the monophyly of *Actinidia* cannot be assessed for the mitochondrial genome due to the low resolution of the mitochondrial data.

None of the four sections forms a monophyletic group in our study, confirming the findings of Li et al. (2002). *Strigosae* being

under-represented in the present study, no definite conclusion can be drawn in that case. Concerning *Maculatae* and *Stellatae*, constraint analyses revealed that an extra 34 and 33 steps, respectively, are needed to force these two well-represented sections to be monophyletic. Even members of the morphologically distinctive *Leiocarpae* section (Huang et al. 1999; He et al. 2000) do not form a natural group. As already suggested (Testolin et al. 1997; Li et al. 2002), *A. rufa*, formerly placed in the same section as *A. arguta* by Dunn (1911), should better be excluded from the *Leiocarpae* section based on the information provided by nuclear (Huang et al. 1997; Testolin and Ferguson 1997), chloroplast (Huang et al. 1997; Cipriani et al. 1998) and mitochondrial (present study) genomes. Even if this particular taxon is ignored, the *Leiocarpae* section remains paraphyletic in each of the four equally parsimonious chloroplast trees. Nevertheless, the paraphyly of *Leiocarpae* is not strongly supported. First, in both heuristic and exhaustive searches, one of the equally parsimonious trees displays a monophyletic serie *Lamellatae*. Second, serie *Solidae* would need at least three additional steps to achieve monophyly. However, by contrast with the chloroplast data, the mitochondrial data support unambiguously the hypothesis of a close relationship between the three *Solidae* species (mitotype C is synapomorphic for *A. macrosperma*, *A. polygama* and *A. valvata*). The allopolyploid origin of the two tetraploid varieties of *A. macrosperma* (Li et al. 2002) probably contributes to obscure the relationships within this serie *Solidae*. Third, once serie *Solidae* is constrained to be monophyletic, no additional step is required for the entire section *Leiocarpae* to be monophyletic.

In conclusion, chloroplast sequences, and to a lesser extent mitochondrial indels, provide clear evidence of conflicts between morphological classifications and molecular phylogenies. This suggests that the infrageneric classifications that have been erected in the past do not reflect the molecular evolutionary history of the *Actinidia* species. Likewise, based on the nuclear, chloroplast and mitochondrial molecular data currently available in *Actinidia*, we evaluate that more than one-quarter of the living taxa has experienced at least one episode of hybridization at some point of their evolutionary history. However, some reticulation events may have been missed due to a lack of molecular resolution, particularly in respect to the mitochondrial data. It is probable that the relatively high levels of species interfertility, leading to allopolyploid but perhaps also homoploid hybridizations, result in unclear species boundaries and account for the taxonomic confusion presently recognized within the genus.

Compléments de discussion

Un processus d'autopolyploïdisation à l'origine des cytotypes 4x d'*A. chinensis* et 6x d'*A. deliciosa* ?

Toutes les données moléculaires obtenues à ce jour sur les deux espèces cultivées *A. chinensis* et *A. deliciosa*, qu'elles soient issues du génome nucléaire ou chloroplastique, confortent les études taxonomiques qui concluaient à une étroite parenté (Crowhurst *et al.* 1990; Crowhurst and Gardner 1991; Cipriani and Morgante 1993), et identifiaient *A. chinensis* comme un probable ancêtre d'*A. deliciosa* (Crowhurst *et al.* 1990). L'origine de l'hexaploïde *A. deliciosa* reste cependant controversée : certaines données moléculaires suggèrent une origine autopolyploïde avec *A. chinensis* comme seul ancêtre d'*A. deliciosa* (Testolin and Ferguson 1997) tandis que d'autres confortent l'hypothèse d'une origine allopolyploïde avec au moins deux espèces ancêtres (Crowhurst *et al.* 1990; Crowhurst and Gardner 1991).

La production de gamètes non réduits est actuellement considérée comme la voie de doublement chromosomique la plus fréquente dans la nature (Ramsey and Schemske 1998) :

- Les triploïdes apparaissent au sein d'une population diploïde suite à l'union de deux gamètes, l'un réduit et l'autre pas.
- Les tétraploïdes apparaissent au sein d'une population diploïde suite à l'union de deux gamètes non réduits, ou, alternativement, suite à l'union d'un gamète non réduit provenant d'un triploïde avec un gamète réduit provenant d'un diploïde.
- Pour les hexaploïdes, trois scénarios sont possibles. Le premier consiste en un croisement entre deux tétraploïdes, l'un des deux gamètes étant non réduit. Les hexaploïdes peuvent aussi résulter d'un croisement entre un diploïde et un tétraploïde, ou d'un croisement entre deux triploïdes, si les deux gamètes sont non réduits.

Nous ne connaissons rien sur la genèse en milieu naturel des *A. chinensis* tétraploïdes, l'émergence éventuelle de triploïdes, ou la formation possible d'hybrides fertiles entre les divers cytotypes. Toutefois, en condition expérimentale, des croisements entre cytotypes d'*A. chinensis* ont abouti à la production de gamètes non réduits (Yan *et al.* 1997b) et témoignent d'un faible niveau d'interfertilité (Guijun *et al.* 1994). Alors que des diploïdes, triploïdes et tétraploïdes émergent d'hybridations entre diploïdes et tétraploïdes, aucun hexaploïde n'apparaît (Guijun *et al.* 1994; Yan *et al.* 1997b). Parallèlement, tous les descendants de croisements entre tétraploïdes

se révèlent tétraploïdes (Guijun *et al.* 1994; Yan *et al.* 1997b). Bien que les triploïdes soient reconnus comme une des voies majeures de formation des polyploïdes (Ramsey and Schemske 1998), ils ont jusqu'ici été négligés dans toutes les études engagées au sein du genre *Actinidia*. Les *Actinidia* triploïdes produisent pourtant un fort taux de gamètes non réduits (Yan *et al.* 1997b). En résumé, les études expérimentales entreprises jusqu'à présent pour produire des hexaploïdes "synthétiques" ont toutes échoué.

Le fait que le mode de formation des polyploïdes ne soit pas complètement élucidé chez les *Actinidia* n'empêche pas de rechercher les possibles ancêtres diploïdes. De récentes investigations ont été menées ces dernières années pour déterminer le ou les taxons ayant contribué au génome d'*A. deliciosa* mais les preuves irréfutables font encore défaut. En dépit des faibles indices rassemblés, l'idée généralement admise est que la race tétraploïde d'*A. chinensis* a joué un rôle de pont dans la formation d'*A. deliciosa* (Testolin and Ferguson 1997; Yan *et al.* 1997a). Cependant, l'origine des tétraploïdes *A. chinensis* et des hexaploïdes *A. deliciosa* fait encore aujourd'hui l'objet de controverses avec trois scénarios qui s'opposent. Selon le premier, tétraploïdes et hexaploïdes ont pour seul ancêtre *A. chinensis* (Testolin and Ferguson 1997). Le deuxième prévoit un premier événement d'autopolyploïdisation, aboutissant à la race tétraploïde d'*A. chinensis*, suivi par un événement d'allopolyploïdisation, aboutissant à *A. deliciosa* (Huang *et al.* 1997). Le troisième comporte deux événements d'allopolyploïdisation successifs (Atkinson *et al.* 1997). Ces trois scénarios impliquent que respectivement une, deux ou trois espèces distinctes auraient contribué au génome d'*A. deliciosa*.

L'utilisation d'une nouvelle source d'informations phylogénétiques, le génome mitochondrial, fournit un éclairage nouveau sur l'histoire évolutive d'*A. chinensis* et de son apparenté hexaploïde *A. deliciosa*. Concernant *A. chinensis*, l'absence de différenciation entre les deux cytotypes, pour le mitotype comme pour les séquences chloroplastiques, suggère une origine autopolyploïde de la population tétraploïde. Ceci est cohérent avec la distribution géographique des deux cytotypes, la population tétraploïde vivant en sympatrie avec la population diploïde dans la partie centrale de la zone occupée par l'espèce (Guijun *et al.* 1994). L'absence de polymorphisme de longueur des fragments de restriction de l'ADNcp et de l'ADNmt dans la population diploïde échantillonnée ne permet pas de tester l'hypothèse d'une origine multiple, comme cela a été fait pour d'autres groupes de végétaux (Soltis *et al.* 1989; Segraves *et al.* 1999). Cette absence de divergence au niveau moléculaire suggère que la polyploïdisation est un événement intervenu récemment dans l'évolution de l'espèce.

L'histoire évolutive d'*A. deliciosa* est plus difficile à reconstruire. L'absence de polymorphisme entre *A. chinensis* et *A. deliciosa* au

niveau de l'ADNcp suggère que *A. deliciosa* est apparu récemment avec *A. chinensis* comme père. Par contre, la présence d'un polymorphisme au niveau de l'ADNmt associée à une résolution plus faible de la phylogénie mitochondriale conduit à plus d'incertitude concernant la lignée maternelle. *A. deliciosa* héberge trois mitotypes distincts, D, G et H, le premier étant une plésiomorphie partagée par plusieurs espèces d'*Actinidia* mais pas *A. chinensis*, le deuxième étant une apomorphie partagée notamment avec *A. chinensis* et le troisième étant une autapomorphie. La présence de deux mitotypes partagés au sein d'*A. deliciosa* suggère que plusieurs événements de polyploïdisation soient à l'origine de la formation de l'espèce ou que des flux de gènes postérieurs à l'événement de polyploïdisation se sont produits. Une origine multiple au sens de Soltis (1999), modèle valable également pour les espèces autopolyploïdes, n'a rien de surprenant pour une espèce dioïque puisqu'une origine double (un mâle et une femelle) est au minimum nécessaire à la perpétuation d'un nouveau cytotype. De plus, l'hypothèse d'une polyploïdisation dérivant d'événements multiples et indépendants est cohérente avec l'importante diversité révélée par les loci microsatellites nucléaires chez *A. deliciosa* (Chat *et al.* 2002).

La présence du mitotype D chez *A. deliciosa*, plésiomorphie partagée mais jamais observée chez *A. chinensis*, est plus inattendue. L'homogénéité des séquences nucléaires ITS (Li *et al.* 2002) ainsi que l'hérédité de type polysomique des marqueurs nucléaires (Chat *et al.* 2002) confortent l'hypothèse d'une origine autopolyploïde d'*A. deliciosa*. Une origine hybride étant alors exclue pour l'espèce, seules deux hypothèses peuvent être avancées pour expliquer l'absence du mitotype D chez *A. chinensis* et sa présence chez *A. deliciosa* : un biais dans l'échantillonnage d'*A. chinensis* ou une hybridation/introgression mitochondriale touchant une population particulière d'*A. deliciosa*. Deux approches peuvent être proposées pour répondre à cette question. Les régions ITS de l'unique individu *A. deliciosa* détenteur du mitotype D dans notre étude devront être séquencées pour vérifier s'il n'a pas une origine hybride. Dans le cas où l'origine hybride serait infirmée, un échantillonnage au sein des populations d'*A. chinensis* au-delà des limites administratives des trois régions précédemment prospectées, Jiangxi, Hubei et Fujian, devrait permettre d'élucider ce polymorphisme.

A. callosa : un scénario d'allopolyploïdisation déduit des phylogénies nucléaires et chloroplastique (Li *et al.* 2002) mais partiellement invalidé par la phylogénie mitochondriale

Six variétés ont été identifiées à l'intérieur de l'espèce *A. callosa*, trois d'entre elles ont été prises en compte dans notre étude comme dans celle de Li *et al.* (2002). Li *et al.* (2002) ont suggéré que certains des caractères morphologiques rencontrés dans l'espèce puissent

résulter d'introgressions. Profitant du fait que le modèle d'évolution du génome nucléaire est de type additif, ces auteurs ont pu mettre en évidence une origine hybride pour l'une des trois variétés (Tableau 3). Ils déduisent de la phylogénie nucléaire (ITS) que la variété *A. callosa* var. *strigillosa* est un allotétraploïde provenant d'une hybridation entre *A. callosa* et *A. chinensis* et de la phylogénie chloroplastique (*mat*K) que *A. chinensis* a vraisemblablement joué le rôle de père dans le croisement original.

Tableau 3 : Polymorphisme intraspécifique chez *A. callosa* et probable origine allopolyploïde de *A. callosa* var. *strigillosa*.

Espèce	Variété	Ploïdie	cpDNA (Li *et al.* 2002)	ITS nucléaire (Li *et al.* 2002)	mtDNA
A. callosa	*henryi*	2x	clade III	clade III	D
	discolor	2x	clade III	clade III	D
	strigillosa	4x	clade I	clade I + clade III	G
A. chinensis		2x, 4x	clade I	clade I	G
A. deliciosa	*deliciosa*	6x	clade I	clade I	D + G + H

Lors de notre étude sur l'ADNcp, aucune divergence n'est apparue entre les séquences chloroplastiques (*rbc*L et *trn*L-*trn*F) d'*A. callosa* var. *strigillosa* et d'*A. chinensis*, ce qui conforte l'hypothèse d'une filiation entre les deux taxons. Mais nous avons constaté avec surprise que les mitotypes étaient également identiques (Tableau 3), contredisant partiellement le scénario de Li *et al.* (2002). Le processus évolutif conduisant à *A. callosa* var. *strigillosa* est donc probablement beaucoup plus complexe que celui imaginé par Li *et al.* (2002) sur la seule base des données chloroplastiques et nucléaires : ce taxon a probablement enregistré plusieurs réticulations successives. En s'inspirant des scénarios de polyploïdisation issus d'événements multiples et indépendants (Soltis and Soltis 1999), nous avons échafaudé un scénario alternatif à celui de Li et al (2002) qui est compatible avec les informations phylogénétiques provenant des trois génomes nucléaire, chloroplastique et mitochondrial (Figure 10).

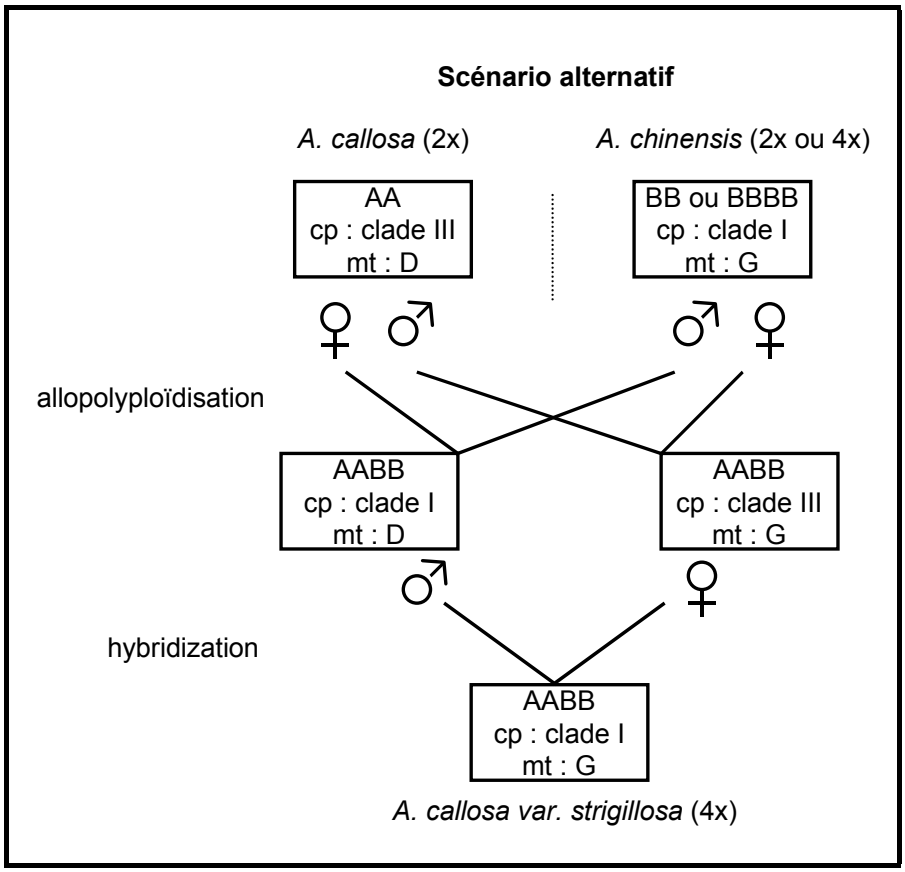

Figure 10 : Possible genèse du taxon *A. callosa* var. *strigillosa*.

Le haut niveau d'interfertilité (plus de 90% des graines contiennent un embryon et un endosperme et plus de 73% germent) (cf Annexe 6 Hirsch *et al.* 2001) entre *A. callosa* et *A. chinensis* et leur distribution sympatrique (Liang 1983) confortent l'hypothèse d'une probable origine récurrente de l'allopolyploïdie entre les deux espèces. D'un point de vue évolutif, ce résultat rejoint ceux obtenus chez *Tragopogon* (Soltis and Soltis 1989) et *Brassica* (Song and Osborn 1993), genres au sein desquels un même allopolyploïde entre deux espèces A et B est rencontré alternativement sur le cytoplasme de l'espèce A ou sur celui de l'espèce B.

Une estimation de la fréquence des événements de polyploïdisation et de réticulation

Le tableau 4 récapitule les informations relatives aux 41 taxons échantillonnés dans notre étude. Le niveau de ploïdie est déduit des comptages chromosomiques et des mesures de cytométrie de flux réalisés préalablement à cette étude. A défaut d'indices contraires, un polyploïde est supposé résulter d'un processus d'autopolyploïdisation. Les indices relatifs à de probables événements d'hybridation sont indiqués : une origine hybride est supposée lorsque les séquences nucléaires ITS sont nettement divergentes (Li *et al.* 2002) et/ou lorsque des incongruences ponctuelles apparaissent entre les

phylogénies chloroplastique et mitochondriale (présente étude). Le taux de taxons polyploïdes est important et comparable au taux de taxons hybrides (environ le quart de notre échantillon). Parmi les polyploïdes, les autopolyploïdes sont deux fois plus nombreux. Le pourcentage de taxons hybrides est très certainement sous-évalué en raison de l'indisponibilité des séquences nucléaires ITS de certains taxons, d'une phylogénie maternelle encore faiblement résolue, et de réticulations successives pouvant masquer l'origine hybride d'un taxon, comme c'est le cas pour *A. callosa* var. *strigillosa* avec les phylogénies chloroplastique et mitochondriale.

Tableau 4 : Processus évolutifs à l'origine des 41 taxons pris en compte dans cette étude et déduits des phylogénies de gènes.

Espèce	Variété	Echantillonnage (Li *et al.* 2002)	Origine hybride	Ploïdie	Origine supposée de la polyploïdie
Section *Leiocarpae*					
A. arguta		Oui		4x	Auto-
A. kolomikta		Oui		2x	
A. melanandra		Oui		4x	Auto-
A. macrosperma	var. *macrosperma*	Oui	Oui (ITS)	4x	Allo-
	var. *mumoides*	Oui	Oui (ITS)	4x	Allo-
A. polygama		Oui		2x	
A. rufa		Oui	Oui (cp/mt)	2x	
A. valvata	var. *valvata*	Oui		4x	Auto-
Section *Maculatae*					
A. callosa	var. *discolor*	Oui		2x	
	var. *henryi*	Oui		2x	
	var. *strigillosa*	Oui	Oui (ITS+cp/mt)	4x	Allo-
A. chrysantha		Oui		4x	Auto-
A. cylindrica	var. *cylindrica*	Non		2x	
	var. *reticulata*	Oui	Oui (ITS+cp/mt)	4x	Allo-
A. glaucophylla	var. *glaucophylla*	Oui		2x	
	var. *rotunda*	Non	Oui (cp/mt)	2x	
A. rubricaulis	var. *coriacea*	Non		2x	
A. sabiaefolia		Oui		2x	
Section *Strigosae*					
A. hemsleyana	var. *hemsleyana*	Oui		2x	
A. melliana		Oui		2x	
Section *Stellatae*					
A. chinensis		Oui		2x,	
A. chinensis		Oui		4x	Auto-
A. deliciosa	var. *deliciosa*	Oui		6x	Auto-
	var. *chlorocarpa*	Non		nd	
A. eriantha	var. *alba*	Oui		2x	
	var. *brunea*	Non	Oui (cp/mt)	nd	
	var. *calvescens*	Non		2x	
	var. *eriantha*	Oui		2x	
A. farinosa		Non		2x	
A. fulvicoma	var. *fulvicoma*	Non		2x	
	var. *lanata*	Oui		2x	
A. grandiflora		Non		4x	Auto-
A. guilinensis		Non		2x	
A. hubeiensis		Non		2x	
A. lanceolata		Non		2x	
A. latifolia	var. *latifolia*	Oui		2x	
A. lijiangensis		Non	Oui (cp/mt)	2x	
A. persicina		Oui	Oui (ITS)	2x	
A. setosa		Non		2x	
A. styracifolia		Oui	Oui (ITS)	2x	
A. zhejiangensis		Oui	Oui (ITS)	2x	
Nombre de taxons			11 (26%)	11 (28%)	7 autopolyploïdes (64%) 4 allopolyploïdes (36%)
Taxons examinés			41	39	11

Commentaires critiques

Lorsque nous avons débuté cette étude, rares étaient les phylogénies de ce niveau taxonomique qui intégraient des données mitochondriales. Nous prenions le risque de ne détecter aucun polymorphisme interspécifique et nous n'aurions jamais parié sur la mise en évidence de polymorphisme intraspécifique, qui plus est chez des espèces autopolyploïdes. La stratégie adoptée qui consistait à initier l'étude par une recherche de polymorphisme par PCR-RFLP a été fructueuse. Cette démarche semble particulièrement adaptée dans le cas du génome mitochondrial compte tenu du type de variations phylogénétiques et de l'actuelle méconnaissance de leur répartition.

A notre connaissance, c'est la première fois que l'opportunité se présentait au sein des angiospermes d'étudier les relations entre espèces par le biais de trois phylogénies basées sur un génome à hérédité maternelle, un génome à hérédité paternelle et un génome à hérédité biparentale. Deux stratégies ont été utilisées chez les *Actinidia*, la stratégie classiquement adoptée chez les plantes et reprise par Li et al (Li *et al.* 2002), qui s'appuie sur les données chloroplastiques et nucléaires, et la nôtre, qui repose sur les données chloroplastiques et mitochondriales. Il est difficile de les comparer en terme de résolution compte tenu de différences dans l'échantillonnage des taxons et dans les techniques employées. Il faut néanmoins souligner leur complémentarité : certains événements d'hybridation précédemment révélés par Li *et al.* (2002), comme par exemple chez *A. macrosperma*, n'ont pu être décelés dans notre étude. A l'inverse, des enseignements inattendus sont tirés de la phylogénie mitochondriale qui contredisent partiellement les hypothèses élaborées précédemment à partir des données nucléaires et chloroplastiques, comme par exemple l'origine du taxon *A. callosa* var. *strigillosa*, mais concordent avec le modèle de polyploïdisation récurrente formalisé par Soltis (1999) et déjà confirmé sur plusieurs dizaines de taxa polyploïdes (Soltis et Soltis 1993).

Conclusion

Nous nous proposions en débutant ce travail de clarifier les relations phylogénétiques entre espèces d'*Actinidia*, de traquer d'éventuelles réticulations et éventuellement d'élucider le processus de polyploïdisation au sein du genre. Nous avions choisi de reconstruire l'histoire évolutive du genre *Actinidia* par le biais des génomes cytoplasmiques, le chloroplaste à hérédité paternelle et la mitochondrie à hérédité maternelle, en utilisant la méthode de parcimonie.

Au moment où nous avons débuté ce travail, l'hérédité contrastée des génomes cytoplasmiques au sein du genre *Actinidia* était connue. Cependant, le mode d'hérédité paternel des chloroplastes n'avait été établi que sur la base de croisements interspécifiques. Ayant réussi à mettre en évidence un polymorphisme chloroplastique au sein de l'espèce cultivée *A. deliciosa*, nous avons confirmé ce mode de transmission, inhabituel chez les angiospermes, sur 143 hybrides intraspécifiques issus de croisements contrôlés. Aucune déviation n'a jamais été constatée sur les quelques 250 individus examinés à ce jour et provenant d'hybridations intra comme interspécifiques. En nous aidant de la loi binomiale, nous avons pu extrapoler ces résultats à l'ensemble de la population théorique des *Actinidia*. Le taux maximum de "fuite" maternelle des chloroplastes et de "fuite" paternelle des mitochondries est estimé à 1.7% et 1.9%, respectivement, pour un risque de deuxième espèce de 1%.

Nous avons poursuivi nos investigations sur le mode de transmission des génomes cytoplasmiques en recherchant d'éventuels cas de transmission atypique. Les deux études que nous avons successivement engagées, l'une concernant une plante hétéroplasmique et l'autre concernant des haploïdes gynogénétiques, nous font soupçonner l'existence d'événements rares de transmission biparentale chloroplastique et de captures chloroplastiques instantanées chez *Actinidia*.

Au début de la thèse, seule une ébauche de phylogénie moléculaire chloroplastique basée sur 17 sites de restriction de l'ADNcp et comportant 16 espèces d'*Actinidia* était publiée (Cipriani *et al.* 1998). Depuis, deux phylogénies moléculaires basées sur des séquences nucléaires (ITS) et chloroplastiques (*mat*K) et comportant 27 taxons ont été publiées (Li *et al.* 2002). Pour notre part, nous avons reconstruit des arbres phylogénétiques à l'aide de deux séquences chloroplastiques (*rbc*L et *trn*L-*trn*F, soit environ 1900 bp)

échantillonnés sur 42 taxons appartenant à 30 espèces différentes. Nous y avons juxtaposé les huit haplotypes mitochondriaux révélés par l'étude des sites de restriction au niveau des deux introns sélectionnés (*nad*1-2/3 et *nad*4-1/2). L'exploitation et la confrontation des deux sources d'information phylogénétique ont fourni des indications fines et inédites sur l'histoire évolutive de ce genre. En premier lieu, du polymorphisme interspécifique et intraspécifique a été détecté, tant au niveau chloroplastique qu'au niveau mitochondrial. En deuxième lieu, la phylogénie paternelle (données chloroplastiques) ne s'oppose pas notablement à la phylogénie maternelle (données mitochondriales), ce qui était pour le moins inattendu compte tenu du mode d'hérédité contrasté des génomes cytoplasmiques dans le genre et de la faculté reconnue des espèces d'*Actinidia* à s'entrecroiser.

La confrontation des trois phylogénies moléculaires, nucléaire, chloroplastique, et mitochondriale, nous a renseigné sur l'importance des événements d'hybridation (au moins 25% des taxons) et de polyploïdisation (environ 25% des taxons, un tiers d'allo- et deux tiers d'autopolyploïdes). Les espèces ou taxons hybrides ont été identifiées sur la base d'une nette divergence des données nucléaires ITS (Li *et al.* 2002) et/ou sur la base d'incongruences ponctuelles entre les phylogénies chloroplastiques et mitochondriales. Le pourcentage de taxons hybrides, homoploïdes ou allopolyploïdes, est très certainement sous-évalué en raison de l'indisponibilité des séquences nucléaires ITS de certains taxons, d'une phylogénie maternelle encore faiblement résolue, et d'une possible succession de réticulations au sein d'un même taxon, comme c'est le cas pour *A. callosa* var. *strigillosa*. L'origine multiple des polyploïdes, scénario formalisé dans les années 90 et aujourd'hui largement admis, nous a aidé à élucider le cas d'un allotétraploïde entre *A. chinensis* et *A. callosa* possédant un cytoplasme (mitochondrie et chloroplaste) caractéristique d'*A. chinensis* au lieu du cytoplasme "mosaïque" ou "cybride" attendu (chloroplaste de l'espèce père et mitochondrie de l'espèce mère).

Concernant les deux espèces domestiquées, *A. chinensis* et *A. deliciosa*, les indices s'accumulent en faveur d'une origine autopolyploïde, bien qu'il ne soit pas exclu que des événements d'hybridation/introgression soient intervenus dans certaines populations.

Les *Actinidia* sont monophylétiques avec les espèces de la section *Leiocarpae* à la base de l'arbre chloroplastique. Deux clades se distinguent nettement sur l'arbre phylogénétique chloroplastique. Même si l'un d'eux rassemble une majorité de taxons de la section *Stellatae* (16 sur 22), aucun ne correspond à une subdivision infragénérique de la classification, ni actuelle ni passée. Les données mitochondriales n'apportent pas plus de crédit à la classification. Nos résultats montrent donc que les critères morphologiques actuellement utilisés dans la classification des *Actinidia*, tels que la présence ou non de lenticelles sur le fruit, une moelle lamellée ou non, des poils

simples ou en étoile, ne sont pas utilisables pour délimiter des groupes naturels. Il est possible que cette nette incongruence entre "gene trees" et "species trees" soit le résultat de caractères morphologiques homoplasiques. Il est cependant plus vraisemblable que les confusions relatives à l'identification des espèces et à leur histoire évolutive trouvent leur origine dans les fréquentes réticulations observées à l'échelle du genre, conduisant à des espèces hybrides ne présentant pas de frontières morphologiques claires les unes par rapport aux autres.

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Annexes

Annexe 1 : Les quatre principales classifications du genre *Actinidia*

Dunn (1911) 24 species	Li (1952) 36 species	Liang (1983) 53 species	Cui et al (2002) 57 species
Section Ampulliferae	Section *Leiocarpae*	Section *Leiocarpae*	Section *Leiocarpae*
		Serie *Lamellatae*	Serie *Lamellatae*
A. Giraldii	*A. arguta*	*A. arguta*	*A. arguta*
A. melanandra	*A. hypoleuca*	*A. globosa*	*A. globosa*
A. polygama	*A. kolomikta*	*A. henanensis*	*A. henanensis*
A. rufa	*A. kwangsiensis*	*A. kolomikta*	*A. kolomikta*
A. tetramera	*A. maloides*	*A. maloides*	*A. maloides*
A. valvata	*A. melanandra*	*A. melanandra*	*A. tetramera*
	A. polygama	*A. tetramera*	*A. melanandra*
	A. purpurea	Serie *Solidae*	Serie *Solidae*
	A. tetramera	*A. macrosperma*	*A. macrosperma*
Section *Leiocarpae*	*A. valvata*	*A. polygama*	*A. polygama*
A. kolomikta		*A. valvata*	*A. valvata*
Section *Maculatae*	Section *Maculatae*	Section *Maculatae*	Section *Maculatae*
A. callosa	*A. asymmetrica*	*A. callosa*	*A. callosa*
A. coriacea	*A. callosa*	*A. chrysantha*	*A. chrysantha*
A. rubricaulis	*A. coriacea*	*A. cylindrica*	*A. cylindrica*
	A. fortunatii	*A. fasciculoides*	*A. fasciculoides*
	A. glabra	*A. glauco-callosa*	*A. glauco-callosa*
	A. pilosula	*A. glaucophylla*	*A. glaucophylla*
	A. rubricaulis	*A. gracilis*	*A. gracilis*
	A. sabiaefolia	*A. indochinensis*	*A. indochinensis*
	A. trichogyna	*A. laevissima*	*A. laevissima*
	A. venosa	*A. leptophylla*	*A. leptophylla*
		A. rubricaulis	*A. rubricaulis*
		A. sabiaefolia	*A. sabiaefolia*
		A. trichogyna	*A. trichogyna*
		A. ulmifolia	*A. ulmifolia*
		A. umbelloides	*A. umbelloides*
		A. venosa	*A. venosa*

Les quatre principales classifications du genre *Actinidia* (suite)

Dunn (1911) 24 species	Li (1952) 36 species	Liang (1983) 53 species	Cui et al (2002) 57 species
Section *Vestitae*	Section *Stellatae*	Section *Stellatae*	Section *Stellatae*
		Serie *Perfectae*	Serie *Perfectae*
A. Championi	*A. chinensis*	*A. chinensis*	*A. chinensis*
A. chinensis	*A. eriantha*	*A. cinerascens*	*A. cinerascens*
A. Davidii	*A. fulvicoma*	*A. eriantha*	*A. deliciosa*
A. eriantha	*A. kiusiana*	*A. farinosa*	*A. eriantha*
A. fulvicoma	*A. lanceolata*	*A. fulvicoma*	*A. farinosa*
A. Hemsleyana	*A. latifolia*	*A. lanceolata*	*A. fulvicoma*
A. Henryi	*A. longicauda*	*A. latifolia*	*A. lanceolata*
A. holotricha		*A. liangguangensis*	*A. latifolia*
A. lanata		*A. rufotricha*	*A. liangguangensis*
A. lanceolata		*A. styracifolia*	*A. rufotricha*
A. pachyphylla		*A. suberifolia*	*A. setosa*
A. rudis		*A. zhejiangensis*	*A. styracifolia*
A. strigosa			*A. suberifolia*
		Serie *Imperfectae*	Serie *Imperfectae*
		A. grandiflora	*A. grandiflora*
		A. obovata	*A. guilinensis*
		A. pilosula	*A. lijiangensis*
		A. sorbifolia	*A. pilosula*
		A. stellato-pilosa	*A. obovata*
			A. sorbifolia
			A. stellato-pilosa
			A. zhejiangensis
Not classified	Section *Strigosae*	Section *Strigosae*	Section *Strigosae*
A. Fortunati	*A. arisanensis*	*A. carnosifolia*	*A. carnosifolia*
	A. hemsleyana	*A. chengkouensis*	*A. chengkouensis*
	A. henryi	*A. fortunatii*	*A. fortunatii*
	A. holotricha	*A. hemsleyana*	*A. hemsleyana*
	A. melliana	*A. henryi*	*A. henryi*
	A. petelotii	*A. holotricha*	*A. holotricha*
	A. rubus	*A. melliana*	*A. melliana*
	A. rudis	*A. rubus*	*A. rubus*
	A. strigosa	*A. rudis*	*A. rudis*
		A. vitifolia	*A. vitifolia*

Annexe 2 : Les caractères morphologiques servant de base à la classification (Ferguson 1990b)

A Feuille glabre ou légèrement pubescente
B Fruit non maculé……**Sect. *Leiocarpae*** (Dunn) Li
C Moelle lamellée……Ser. *Lamellatae* C.F. Liang
CC Moelle non lamellée……Ser. *Solidae* C.F. Liang

BB Fruit maculé……**Sect. *Maculatae*** Dunn

AA Feuille et branche très poilue ou laineuse
(Sect. Vestitae Dunn)
D Poil simple, dur, gros……**Sect. *Strigosae*** Li

DD Poils doux et fins, stellés sur la face inférieure
des feuilles……**Sect. *Stellatae*** Li
E Face inférieure de la feuille couvert de poils
stellés persistants et denses……Ser. *Perfectae* C.F. Liang
EE Face inférieure de la feuille, poils
imparfaitement stellés ou poils stellés
caduques épars……Ser. *Imperfectae* C.F. Liang

Annexe 3 : Table 2 supplied as supplementary material of "A one step chloroplast capture in kiwifruit" :

Table 2: Probability to observe the occurrence of four trihaploids of genotype [-] considering syngamy followed by random elimination of three sets of chromosomes among the six ones inherited by the zygote (2n=6x).

Locus		No. of	Probability		
No. (l)	Name	paternal-specific alleles per locus (n)	of the zygote (2n=6x) to inherit i paternal-specific alleles (P_{il})	of the trihaploid to be [-] after random elimination of 3 chromosomes (Q_{il})	of one trihaploid to be [-] for all the n paternal-specific alleles at the l locus ($P_{il} \times Q_{il}$)
1	UDK96-001	1	i=0: 1/2	1	1/2
			i=1: 1/2	1/2	1/4
					$\sum_{i=0}^{i=n}(P_{il} \times Q_{il}) = 3/4$
2	UDK96-030	3	i=0: 1/20	1	1/20
			i=1: 9/20	1/2	9/40
			i=2: 9/20	4/20	36/400
			i=3: 1/20	1/20	1/400
					$\sum_{i=0}^{i=n}(P_{il} \times Q_{il}) = 147/400$
3	721	4	i=1: 1/5	1/2	1/10
			i=2: 3/5	4/20	12/100
			i=3: 1/5	1/20	1/100
					$\sum_{i=0}^{i=n}(P_{il} \times Q_{il}) = 23/100$
4	722	3	i=0: 1/20	1	1/20
			i=1: 9/20	1/2	9/40
			i=2: 9/20	4/20	36/400
			i=3: 1/20	1/20	1/400
					$\sum_{i=0}^{i=n}(P_{il} \times Q_{il}) = 147/400$
Probability for the four trihaploids to be [-] for all the 11 paternal-specific alleles at the four SSR loci examined: $\left(\prod_{l=1}^{l=4}\sum_{i=0}^{i=n} P_{il} \times Q_{il}\right)^4 = 2.9 \times 10^{-7}$					

Annexe 4 : A. M. Hirsch, R. Testolin, S. Brown, J. Chat, D. Fortune, J. M. Bureau, D. De Nay 2001 Embryo rescue from interspecific crosses in the genus *Actinidia* (kiwifruit). Plant Cell Reports 20:508-516.

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CELL BIOLOGY AND MORPHOGENESIS

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Embryo rescue from interspecific crosses in the genus *Actinidia* (kiwifruit)

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Abstract Within the framework of a broad program of interspecific crosses involving different *Actinidia* species and ploidy races, we carried out an assay of interspecific hybridization using a small number of the *Actinidia* species available in European repositories. Our aim was to conduct a preliminarily assessment of the degree of crossability occurring in the genus and the effectiveness of different techniques and media in rescuing interspecific hybrids. A set of culture media was developed to replace the hybrid embryo's deficient endosperm to ensure survival of the embryos at the globular and heart stages. In several crosses immature embryos that reached the torpedo stage were rescued when incubated in the proper media. The embryo rescue was the unique tool which led to hybrid plantlets for four crosses: *A. kolomikta* × *A. chinensis*, *A. polygama* × *A. valvata*, *A. arguta* Issa × *A. polygama* and *A. kolomikta* × *A. deliciosa*. Flow cytometry was used to check the ploidy level of the parental genotypes and the progeny. A wide interspecific crossability was found in *Actinidia* that involved species belonging to the different sections on the basis of traditional taxonomy.

Keywords *Actinidia* · Kiwifruit · Interspecific hybridization · Embryo rescue · Flow cytometry

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Abbreviations *A*: *Actinidia* · *CV*: Coefficient of variation · *C*: Quantity of DNA corresponding to the haploid complement · *G1*: Gap 1 corresponding to the fundamental stage of the proliferation cycle · *G0*: Gap 0; as for G1 in a quiescent cell

Introduction

The world production of kiwifruit relies upon a single species of the genus *Actinidia*, namely *A. deliciosa*. Although quite a large variability in quantitative traits has been found in kiwifruit, many interesting agronomic characters that are lacking in kiwifruit itself are present in the vast genetic resources within the genus *Actinidia*, which encompasses more than 60 species and 100 taxa (Ferguson 1990). For example, flesh colors different from the green one of the kiwifruit can be found in related species, such as *A. chinensis*, which has races with yellow flesh, and in unrelated species, such as those of the section Leiocarpae series Solidae, which have a brilliant orange flesh. Another interesting agronomic trait is the adaptation to cold climates typical of, for example, *A. kolomikta*, a species with a wide distribution up to the Siberian steppes.

The interspecific cross is a traditional tool used to introgress characters of interest into a given crop species. The genus *Actinidia* is reported as being prone to cross easily in the wild (Ferguson 1984). However, despite the number of successful crosses that have been achieved between different species, many attempts to produce plantlets from crosses between species of potential agronomic value have been unsuccessful as evidenced by Pringle (1986), Mu et al. (1990), Ke et al. (1991) and Wang et al. (1994). In many of these unsuccessful crosses, the kiwifruit was one parent. Failures have often been attributed to the unbalanced ploidy level of the parents, since ploidy levels ranging from 2x (=58) to 6x are present in the genus and ploidy races can be found within several species (Ferguson et al. 1997). In *Actinidia*, as in many other genera, crosses between different ploidy lev-

els are more recalcitrant in giving plantlets than crosses carried out using species at the same level of ploidy. Unfortunately, *A. deliciosa*, or kiwifruit, is hexaploid (2n=6x=174) and, in addition, the commercial cultivar, Hayward, is frost-sensitive, whereas most of the remaining species of the genus, including the frost-resistant ones, are diploid or tetraploid (Blanchet and Chartier 1991). All these data explain why it is so difficult to obtain successful interspecific hybrids in the genus *Actinidia*. A new but difficult biotechnological method involving protoplast fusion was recently proposed (Xiao and Hirsch 1996, 1997) with the aim of overcoming these difficulties in carrying out successful interspecific crosses.

In interspecific crosses that have failed, the failure can be attributed to different factors. In some cases fruit set did not occur, or when it occurred, none of the seeds survived, appearing to be empty of embryos and of endosperm. In other crosses, there was only a partial failure at successive points: the formation of the cotyledonary hybrid embryo was inhibited and embryo development stopped at early stages, i.e. at the globular, heart and torpedo stages. In yet other cases, the endosperm was deficient, unable to provide enough nourishment to the embryo until the mature cotyledonary stage could be attained. Therefore, in the genus *Actinidia*, it is of interest to develop artificial culture media able to replace the deficient endosperm in order to facilitate complete hybrid embryo development.

The present paper reports an assay of interspecific hybridization that was carried out using a small number of the *Actinidia* species available in the European repositories. Our aim was to carry out a preliminarily assessment of the degree of crossability occurring in the genus and the effectiveness of different techniques and media in rescuing interspecific hybrids.

Materials and methods

Materials

The *Actinidia* species available in the repositories of the INRA of Bordeaux (France) and the University of Udine (Italy) and used in the mating design are listed in Table 1. Only one sex was available for some species, and this prevented us from including many combinations of species and several reciprocal crosses in the work plan. The different *Actinidia* species used in our program belong to the four sections of the genus (Leiocarpae, Maculatae, Strigosae and Stellatae), and the ploidy level, as estimated by flow cytometry, and average DNA content of each species (in picograms) are also listed in Table 1.

The *Actinidia* species were either diploid, tetraploid or hexaploid. Two species had a polyploid series, namely diploid and tetraploid for *A. chinensis*, diploid, tetraploid and hexaploid for *A. arguta* (Blanchet et al. 1992).

Cross-pollination

Shoots carrying female flowers were bagged with paper bags when the flowers were at pop-corn stage, that is the stage where petals become visible. Paper bags were opened just enough to allow us to operate inside. Flowers too mature and those not yet open were removed. The remaining flowers were pollinated by gently applying pollen with a fine-haired brush or by rubbing fresh male flowers, collected from bagged shoot at crossing time against the female flowers. Pollen collected in advance from early-flowering genotypes was kept stored at 5°C for no longer than 2 weeks. After pollination, the bags were quickly sealed and removed 2 weeks thereafter.

Many crosses were performed at the University of Udine, but only those crosses that resulted in fruit set are discussed in this paper. Other crosses were carried out at the INRA station at Bordeaux.

Table 1 Systematic position[a] and estimation by flow cytometry of the ploidy levels and 2C DNA content in the *Actinidia* species used in interspecific hybridizations

Taxon	Ploidy level (x)	2C DNA content[b] (pg)
1. Section Leiocarpae		
Series Lamellatae		
A. arguta (Sieb. and Zucc.) Planch.	4	3.33
A. arguta var. Issa	6	4.96
A. kolomikta (Maxim. and Rupr.) Maxim.	2	1.49
A. melanandra Franch.	4	3.27
Series Solidae		
A. polygama (Sieb. and Zucc.) Maxim.	2	1.63
A. valvata Dunn	4	3.30
2. Section Maculatae		
A. callosa Lindl.	2	1.45
A. chrysantha C.F. Liang	4	3.43
3. Section Strigosae		
A. hemsleyana Dunn	2	1.63
4. Section Stellatae		
A. eriantha Benth.	2	1.65
A. chinensis Planch.	2 and (4)	1.60
A. deliciosa (A.Chev.) Liang and Ferguson	6	4.90
A. lanceolata Dunn	2	1.54

[a] List of *Actinidia* taxa according to the Liang (1984) classification, revised by Ferguson (1990)
[b] Estimated using Hoechst 33342, without a correction for base composition, relative to the internal standard, *Petunia*

Nutritional culture media for embryo rescue

The media used in this study are presented in Table 2. A first set of media (EbH1, EbH2)was established for the survival of young embryos; a second set (EbG1, EbG2 and EbG2B) was designed to allow the germination of more highly developed embryos. All media were autoclaved 20 min at 110°C, with the pH adjusted to 5.6 before autoclaving.

Fruit sterilization and embryo rescue technique

Immature fruits

These are abscising fruits that have fallen during the 14 days following fruit set and fruits harvested 6 weeks after fruit set. After fruit decontamination with 70% ethanol, the embryos, if any, were aseptically removed under a microscope in a laminar flow cabinet and incubated in 50-mm-diameter petri dishes containing one of the media of the first series.

Table 2 Composition of the nutritional media designed for embryo rescue (weight l^{-1})

Ingredients	EbH1 medium[a]	EbH2 medium[b]	EbG1 medium[c]	EbG2 medium[d]	EbG2B medium[d]
MS mineral solution[f]	MS	MS	MS	1/2-MS	MS
Gamborg's B5 vitamin sol.[e]	B5	B5	B5	B5	B5
Casein hydrolysate (mg)	400	400	400	400	400
Glycine (mg)	2	2	2	2	2
Glutamine (mg)	500	500	500	500	500
Serine (mg)	30	30	30	30	30
Biotin (mg)	1	1	1	1	1
Maltose (g)	30	–	–	–	–
Sucrose (g)	–	30	30	30	30
Mannitol (g)	–	50	–	–	–
Zeatin (mg)	–	1	–	–	–
1-Naphthalene-acetic acid (mg)	–	0.5	–	–	–
Gibberellic acid (GA_3) (mg)	–	–	1	–	–
Agarose (g)	7	7	7	7	7

[a] EbH1: Medium for globular and heart-shape stages
[b] EbH2: Medium for torpedo stage
[c] EbG1: Medium for pre-cotyledonary stage
[d] EbG2, EbG2B: Media for cotyledonary stage development
[e] Reference: Gamborg et al. (l968)
[f] Reference: Murashige and Skoog (l962)

Eight-week-old or older mature fruits

Seeds were extracted from fruits in Udine (Italy) and sent to Paris (France) within a few days. They were sterilized in the laboratory following this procedure: (1) 3 min in a mixture [50/50 (v/v)] of ethanol/Mercryl, a pharmaceutical mixture of sodium laurylsulfate and mercurobutol (Menarini France, Rungis); (2) 30 min in a Cryptonol solution (25 ml l^{-1}), a commercial hydroxyquinoleine (3); 10 min in calcium hypochlorite (30 g l^{-1}; 70 Gay-Lussac grades) followed by three rinses with sterile water.

After dissection under a binocular microscope, embryos and endosperms were separated. Depending on their development stage, the embryos were seeded in petri dishes containing a medium belonging to either the first or the second series.

After plating, the embryos were first allowed to incubate under red light (λ=655±20 nm, E=1.1 mmol m^{-2} s^{-1} for 2 weeks at 25°C), then transferred to continuous white light (λ=660 nm, E=12 mmol m^{-2} s^{-1}). As soon as the plantlets reached 25 mm in length with a well-developed root system they were transplanted into 125-mm-long and 25-mm-diameter tubes containing a paper bridge and a liquid medium (EbG1 without agar) and placed under a 16-h (white light): 8-h (dark) photoperiod.

Determination of the ploidy level in hybrid plantlets by flow cytometry

Leaves and/or roots were sampled aseptically. Cytometric determinations were performed on an EPICS V cytometer (Beckman-Coulter) with an argon laser 2025-05 (Spectra-Physics) following Brown et al. (1991) as adapted to the genus *Actinidia* by Blanchet et al. (1992). Leaves of *Actinidia* spp. and the reference were chopped together in 50 m*M* glucose, 15 m*M* NaCl, 12 m*M* KCl, 5 m*M* Na_2EDTA, 50 m*M* sodium citrate, 0.5% (w/v) Tween 20, 50 m*M* Hepes pH 7.2 and 5 m*M* sodium metabisulfite [Marie and Brown (1993) modified with sodium metabisulfite from stocks stored at –20°C, replacing β-mercaptoethanol, a health hazard]. The reductant was active for several hours. The internal reference for each measurement was either leaf nuclei of *Petunia hybrida* (cv. PxPP6), as in our previous studies (Blanchet et al. 1992), or *A. deliciosa* cv. Hayward. The DNA stain used was bisbenzimide Hoechst 33342 (Aldrich), which was added at a concentration of 7.5 µg ml^{-1} after 0.5 ml of chopped suspension had been filtered through 30-µm nylon filtrettes (Marie and Brown 1993). Hoechst is convenient for rapid, clean ploidy analyses, however, it is sensitive to DNA base composition (Godelle et al. 1993). Our internal reference was *Petunia* with 41.0% GC content (Marie and Brown 1993), while *Actinidia* spp. have a mean GC content of 39.3% (Blanchet et al. 1992). Our DNA calculations are therefore systematically biased towards a slight (15%) overestimation.

Results and discussion

Development of embryos of parental species

Effective media for embryo survival were developed to support the different developmental stages of embryos of the frost sensitive-species, *A. deliciosa*, and those of a frost-resistant species, *A. arguta* var. arguta. Rich culture media are especially needed for the young embryo's stages of development (globular, heart and torpedo). Mineral salts, which are essential components, and several growth-promoting substances are needed in the culture media. For many years, we have used Murashige and Skoog's medium (MS; 1962) as our basic medium for culturing embryos and endosperm of different species of *Actinidia*: full strength for *A. deliciosa* and half-strength mineral salts for several other species, among them *A. arguta*. Particularly important are the sugars that serve as a carbon source and as osmotic stabilizers. Sucrose (3%) was used for all developmental stages of embryos (Table 3), except for the globular and heart stages for which maltose is required in the *Actinidia* genus (Mu et al. 1990). A high osmotic concentration of the medium prevents the precocious germination of young embryos, notably at the torpedo stage, and supports a normal embryonic growth, which is why 5% mannitol was added to the EbH2 medium. Growth regulators were used very sparingly. No hormone was added at the earliest stages of development, the globular and heart stages (EbH1). Auxin action is linked with cell permeability and ion uptake; cytokinin acts on growth (Sharma et al. 1996). Consequently, 1-naphthaleneacetic acid (NAA) and zeatin (Z) were added only at low concentrations to EbH2, and this medium only used to ensure the survival of the torpedo stage. Gibberellic acid (GA_3), which acts on embryo germination, was only added to the EbG1 medium used for the precotyledonary stage. As soon as cotyledonary primordia are initiated, GA_3 is no longer necessary. Amino acids, vitamins and casein hydrolysate were systematically added to the culture media, but these natural adjuvants are not absolutely necessary after the initiation of the cotyledonary primordia.

Table 3 Recovery of viable plantlets from embryos of two parent species, *A. deliciosa* and *A. arguta*, incubated 4–14 weeks after pollination

Medium	Growth regulators[a] (mg l^{-1})			Sugar concentration[b] (g l^{-1})			Number of embryos incubated	Developmental stage of embryos[c]	Weeks after pollination	Number of surviving embryos after 3 weeks of incubation	Percentage of surviving embryos	Number of plantlets recovered	Percentage of recovery
	NAA	Z	GA_3	M	S	Mn							
A. deliciosa													
EbH1	0	0	0	30	0	0	40	g+h	5–6	27	67.5	0	0
EbH2	0.5	1	0	0	30	50	48	t	6–7	35	80.2	0	0
EbG1	0	0	1	0	30	0	110	pc	8–12	91	82.7	58	52.7
EbG2B	0	0	0	0	30	0	100	c	12–14	88	88	72	72
A. arguta													
EbH1	0	0	0	30	0	0	38	g+h	6	23	60.5	0	0
EbH2	0.5	1	0	0	30	50	44	t	6–7	28	63.6	0	0
EbG1	0	0	1	0	30	0	108	pc	10	89	82.4	62	57.4
EbG2	0	0	0	0	30	0	126	c	10–12	101	80.1	95	75.3

[a] *NAA* 1-Naphthaleneacetic acid, *Z* zeatin, *GA_3* gibberellic acid
[b] *M* Maltose, *S* sucrose, *Mn*, mannitol
[c] *g+h* Globular (g) and heart-shape stage (h) of embryo; *t* torpedo stage of embryo, *pc* precotyledonary stage of embryo; c, cotyledonary stage of embryo

The data in Table 3 shows that our culture media allowed a very good survival of young embryos. After 21 days of incubation, the earliest stages of the embryos' development yielded a very good survival percentage in both parental species: about 60% for globular and heart-shaped embryos and about 80% for torpedo embryos. No browning was observed on embryos incubated 3 weeks on EbH1 and EbH2 despite the fact that no anti-browning substance, like polyclar, had been added to these media. On the other hand, neither EbH1 nor EbH2 allowed the recovery of viable plantlets from the earliest stages of embryo development, probably due to the absence of endosperm enzymatic systems essential to the development of the young embryonic stages in the genus *Actinidia*. Mu et al. (1990) needed a nursing endosperm system to obtain the recovery of a few viable plantlets from heart-shaped and torpedo embryos in *A. chinensis*. This type of nursing system was not possible in our breeding program because of the numerous crosses that we carried out. High levels of budding calli were produced in both *A. deliciosa* and *A. arguta* when globular and heart-shaped embryos were incubated between 2 and 5 weeks after pollination on modified EbH2 medium containing NAA and Z but deprived of mannitol. From these calli, somaclonal variants, such as octoploids (2n=8x),were easily isolated. The tendency of embryo to callus, especially at the earliest globular stage, even within the endosperm, was also noticed by Mu et al. (1990) for *A. chinensis*.

The more mature embryos from both species incubated just before initiation of the cotyledonary primordia on EbG1 had a good survival percentage, and the percentage of viable plantlet recovery exceeded 50% in both species. Once the cotyledonary primordia were initiated, a medium deprived of GA_3 (EbG2B for *A. deliciosa* and EbG2 for *A. arguta*) was effective for an easy and rapid germination of embryos (no more than 2 weeks after incubation). In both *A. deliciosa* and *A. arguta* the endosperm showed structural changes between the 7th and the 10th week of embryo development, changing from a viscous form into a solid state.

Embryo rescue in interspecific crosses

Embryo rescue between species having the same ploidy level

The results are presented in Table 4. In such crosses, embryo rescue was realized for embryos incubated after the torpedo stage, at the moment preceding initiation of the cotyledonary primordia. For many hybrid embryos, this incubation occurred at the 6th week after pollination on EbG1 medium. The emergence of the cotyledonary primordia on EbG1 made a rapid transfer from EbG1 to EbG2 or EbG2B media devoid of growth factors possible, and the germination of viable hybrid plantlets took place immediately. Viable hybrid plantlets can be obtained more rapidly by embryo rescue than by the conventional method because in the latter a long period of dormancy is necessary before the seeds can be soaked. With the embryo rescue technique, interspecific hybridization was successful 4–5 months earlier than by the conventional method. This former is a unique tool to obtain the interspecific hybridization between *A. kolomikta* × *A. chinensis* (U 35).

The data presented in Table 4 are a summary of the results of interspecific crosses carried out using parents of the same ploidy level. Of the eight combinations of pairs of diploid species assayed four (50%) showed easy cross-

Table 4 Embryo rescue in controlled interspecific crosses involving species of the same ploidy (*nt* not tested)

Cross code	Cross[a]	Date of fruit harvest or abscission	FS-BF[b]	Number of seeds extracted	Number of seeds dissected	Empty seeds (%)	Seeds with only endosperm[c] (%)	Seeds with embryo + endosperm (%)	Rescued embryos[d] (%)	Ploidy level[e] (x)
Diploid × diploid										
U52	*A. chinensis* × *A. callosa* #54.1 #103	18-10-94	2-2	854	54	1.85	5.55 (S.En)	92.6	92	2
U53	*A. chinensis* × *A. callosa* #54.20 #103	18-10-94	9-9	2,031	75	4.0	5.3 (S.En)	90.7	89	2
U85	*A. chinensis* × *A. callosa* #54.16 #103	23-10-95	4-5	Many[f]	144	2.8	2.1 (S.En)	95.1	73	nt
U80	*A. chinensis*.× *A. eriantha* #54.16 #105.5	23-10-95	5-5	Many	166	6.0	3.6 (S.En)	90.4	71	nt
U68	*A. eriantha* × *A. callosa* #105.2 #103	18-10-94	1-3	332	75	14.6	1.3 (S.En)	84.1	82	2
U93	*A. eriantha* × *A. callosa* #105.2 #103	03-11-95	6-6	Many	71	0	2.8 (S.En)	97.2	83	nt
U96	*A. eriantha* × *A. callosa* #105.4 #103	03-11-95	6-6	Many	100	7.0	2.0 (S.En)	91.0	88	nt
U69	*A. eriantha* × *A. chinensis* #105.2 #54.19	21-09-94	3-3	1,067	85	4.7	0	95.3	95	2
U95	*A. eriantha* × *A. chinensis* #105.4 #54.19	03-11-95	5-5	Many	72	19.4	5.5 (S.En)	75.1	76	nt
U94	*A. eriantha* × *A. lanceolata* #105.2 #83.4	03-11-95	5-9	Many	40	25.0	0	75.0	80	nt
U97	*A. eriantha* × *A. lanceolata* #105.4 #83.4	03-11-95	3-6	Many	49	16.3	0	83.7	80	nt
U91	*A. eriantha* × *A. polygama* #105.2 #70	03-11-95	5-5	Many	69	86.9	4.3 (V.En)	8.69	33	nt
U74	*A. hemsleyana* × *A. chinensis* #50 #54.19	18-10-94	1-42	19	11	9.1	9.1 (V.En)	81.8	81	2
U101	*A. hemsleyana* × *A. chinensis* #50 #54.19	03-10-95	7-30	117	24	25.0	8.3 (S.En)	66.6	12.5	nt
U73	*A. hemsleyana* × *A. eriantha* #50 #105	18-10-94	5-51	188	89	5.6	14.6 (S.En)	79.8	63	2
U104	*A. hemsleyana* × *A. eriantha* #50 #105.1	03-10-95	6-20	92	14	0	71.4 (S.En)	28.6	25	nt
U35	*A. kolomikta* × *A. chinensis* #63 #54.14	05-10-93		83	80	62.5	25 (S.En)	12.5	60	2
U63	*A. polygama* × *A. eriantha* #69 #105.5	21-09-94	3-18	178	70	98.5	1.4 (L.En)	0	0	–
U107	*A. polygama* × *A. eriantha* #69 #105.1	01-10-95	8-10	Many	82	96.3	3.6 (L.En)	0	0	–
Tetraploid × tetraploid										
U90	*A. chrysantha* × *A. arguta* #104.6 #68	03-11-95	15-15	Many	49	8.2	0	91.8	84	nt
U89	*A. chrysantha* × *A. valvata* #104.6 #123.1	03-11-95	12-12	Many	57	8.8	1.7 (S.En)	89.5	84	nt
Hexaploid × hexaploid										
U40	*A. arguta* Issa × *A. deliciosa* #66 #1	05-10-93		Many	100	27	14 (S.En)	59	69	6
U41	*A. arguta* Issa × *A. deliciosa* #60 #1	01-10-93		Many	100	35	25 (S.En)	40	85	6
U54	*A. arguta* Issa × *A. deliciosa* #66-10 #78	18-10-94	16-102	688	80	1.25	1.25 (S.En)	97.5	85.9	6
U55	*A. arguta* Issa × *A. deliciosa* #66-19 #78	18-10-94	15-166	612	53	0	0	100	90	6
U56	*A. arguta* Issa × *A. deliciosa* #66-10 #41	18-10-94	19-75	710	21	0	0	100	81	6

[a] Female parent is written first; # is the accession number
[b] *FS* Fruit set, *BF* bagged flowers
[c] *S.En* Solid endosperm, *V.En* viscous endosperm, *L.En* liquid endosperm, *U* pollination in Udine
[d] Rescued embryos were estimated as the percentage of complete seeds (with embryo and endosperm) able to produce viable plantlets
[e] Determined by flow cytometry in plantlets issued from embryo rescue
[f] Many: ≥200

Table 5 Embryo rescue in controlled interspecific crosses involving species of different ploidy (*nt* not tested)

Cross code	Cross[a]	Date of fruit harvest or abscission	FS-BF[b]	Number of seeds extracted	Number of seeds dissected	Empty seeds (%)	Seeds with only endosperm[c] (%)	Seeds with embryo + endosperm (%)	Rescued embryos[d] (%)	Ploidy level[e] (x)
Diploid × tetraploid										
U77	*A. polygama* × *A. valvata* #69 #109.1	13-09-94	15-21	3,685	68	51.4	4.4 (V.En.)	44.1	43	3
B6	*A. polygama* × *A. arguta* #W18-2 #W4	26-09-94	5-3	60	60	80	15 (V.En.)	5	0	–
Tetraploid × diploid										
U88	*A. chrysantha* × *A. polygama* #104.6 #70	03-11-95	5-5	Many[f]	67	26.8	31.3 (V.En.)	41.8	7.1	nt
Diploid × hexaploid										
U37	*A. kolomikta* × *A. deliciosa* #KM1 #M2	05-10-93		115	100	75	25 (V.En.)	0	0	–
U38	*A. kolomikta* × *A. deliciosa* #KM2 #M2	05-10-93		70	60	100	0	0	0	–
B7	*A. kolomikta* × *A. deliciosa* #W30 #K7	30-06-94	1-11	43	43	100	0	0	0	–
B8	*A. kolomikta* × *A. deliciosa* # W12 # K7	30-06-94	1	107	107	100	0	0	0	–
B9	*A. kolomikta* × *A. deliciosa* #W14 #K7	30-06-94	25	1,534	1,534	81.8	17.4 (V.En.)	0.65	20	4; 6
B4	*A. polygama* × *A. deliciosa* #W18–2 #K8	05-93	1	147	140	2.85	97.1 (L.En.)	0	0	–
B5	*A. polygama* × *A. deliciosa* #W18–2 #K7	12-09-94	8-8	993	990	1	99 (L.En.)	0	0	–
Hexaploid × diploid										
B2	*A. deliciosa* × *A. eriantha* cv. Hayward W 19-4	22-10-92	5-5		100	40	27	33	42.4	4
U75	*A. arguta* Issa *x A. polygama* #18-19 #PG2	18-10-94	1-65	2	2	0	0	100	50	nt
Hexaploid × tetraploid										
B1	*A. deliciosa* × *A. chinensis* cv. Hayward W 17	22-10-92	5-5		150	16.6	50.1 (S.En.)	33.3	90	5

[a] Female parent is written first; # is the accession number
[b] *FS* Fruit set, *BF* bagged flowers
[c] *S.En* Solid endosperm, *V.En* viscous endosperm, *L.En* liquid endosperm, *U* pollination in Udine, *B* pollination in Bordeaux
[d] Rescued embryos were estimated as the percentage of complete seeds (with embryo and endosperm) able to produce viable plantlets
[e] Determined by flow cytometry in plantlets issued from embryo rescue
[f] Many: ≥200

ability with regular fruit set, many seeds per fruit and embryos that were normal and easy to rescue. The list of these species include *A. chinensis*, *A. eriantha* and *A. lanceolata* from the section Stellatae and *A. callosa* from the section Maculatae. In all cases, we replicated the cross combination by including the reciprocal and/or using different genotypes for at least one of the two species of the pair. In two combinations (*A. kolomikta* × *A. chinensis* and *A. polygama* × *A. eriantha*), in which species from different sections were involved, fewer fruits were produced, many seeds were empty and embryo rescue was less successful or completely unsuccessful (Table 4).

A. hemsleyana (sect. Strigosae), the only diploid species available, set few fruits when crossed with species of section Stellatae, namely *A. chinensis* and *A. eriantha*, but surprisingly, most seeds were complete with both endosperm and embryo and they were rescued very well.

Among the tetraploids, *A. chrysantha* (sect. Maculatae) showed a tendency to cross easily with two species of section Leiocarpae (*A. arguta* and *A. valvata*).

At the hexaploid level, two accessions of *A. arguta* (sect. Leiocarpae) crossed well with different genotypes of *A. deliciosa*, the kiwifruit, in spite of the great taxonomic distance.

Embryo rescue between species of different ploidy levels

The results of these crosses are summarized in Table 5. In most cases when the parents of interspecific crosses differ in ploidy, the fruits abscise early within 1 or, at the latest, 2 weeks following pollination. For this reason we carried out crosses between genotypes of different ploidy levels to a much lesser extent. As a general rule, these

unbalanced combinations gave a lower fruit set. The few flowers which did set, produced fruits with few seeds, most of which were non-viable, that is empty or with a deficient liquid endosperm, like in the cross *A. polygama* × *A. deliciosa*.

Plantlets were seldom regenerated (Table 5). However, embryo rescue carried out on embryos that had reached the torpedo stage was the exclusive method for obtaining hybrid plantlets in the following crosses: *A. polygama* × *A. valvata* (U77); *A. arguta* Issa × *A. polygama* (U75); *A. kolomikta* × *A. deliciosa* (B9). For two crosses, *A. deliciosa* × *A. eriantha* (B2) and *A. deliciosa* × *A. chinensis* (B1), both methods, the conventional one and embryo rescue, were successful. For the combination *A. kolomikta* × *A. deliciosa*, five crosses were carried out using different accessions, with only one succeeding in embryo rescue (Table 5). The results obtained in the different crosses were independent of the degree of ploidy imbalance and the direction of the cross – that is whether the female parent was at a lower or at a higher ploidy level. The latter result seems noteworthy since Pringle (1986) reported that when interspecific crosses were conducted using parents of different ploidy, better results were obtained by choosing the genotype with the lower ploidy number as the female parent. We do not know whether the failure in obtaining plantlets from these interspecific crosses must be attributed to the taxonomic distance between species or to the chromosome imbalance. Both reasons could be true, although taxonomic distance appears to be the most severe constraint.

DNA ploidy

As previously reported by Blanchet et al. (1992) and Ollitraut-Sammarcelli et al. (1994), *Actinidia* tissue lends itself well to DNA analysis, provided that antioxidants are included in buffers, that the tissue to buffer ratio is low and that chopping is brief. Problems such as base-line debris, sample browning or mucilage formation (from leaf tissue) can thereby be avoided. Furthermore, even a few in vitro roots can give satisfactory nuclear suspensions without the above-mentioned problems – due to secondary products of leaves – which are particularly found in certain species, e.g. *A. arguta*, *A. deliciosa* and *A. polygama*. The average DNA content expressed in picograms is listed for each *Actinidia* species in Table 1. The value of DNA content earlier obtained for *A. deliciosa* (Blanchet et al. 1992) by flow cytometry, 2C=6x=4.19 pg and 38.7% GC, was confirmed by Hopping (1994) by the same technique. The basic ploidy unit, x, is therefore equivalent to 4.19/6 pg, namely 0.7 pg or 670×10^6 base pairs.

As previously, the coefficient of variation of the G0–G1 (2C) nuclei from *Actinidia* spp ranged between 2.7% and 6.0%, measured as the tightness of histogram peaks (Fig. 1). The ratio of 2C peak positions, *Actinidia*/*Petunia* (Fig. 2) or *Actinidia*/Hayward, was used to deduce the "DNA ploidy level". This was highly reproducible. For example, 18 results from tetraploids scattered through 100 samples over 2 days showed 3.6% variation (100 × the standard deviation/mean). The term "DNA ploidy" is considered to be appropriate to distinguish this diagnosis based upon genome size from the classical cytogenetic determination of ploidy. Figure 1A

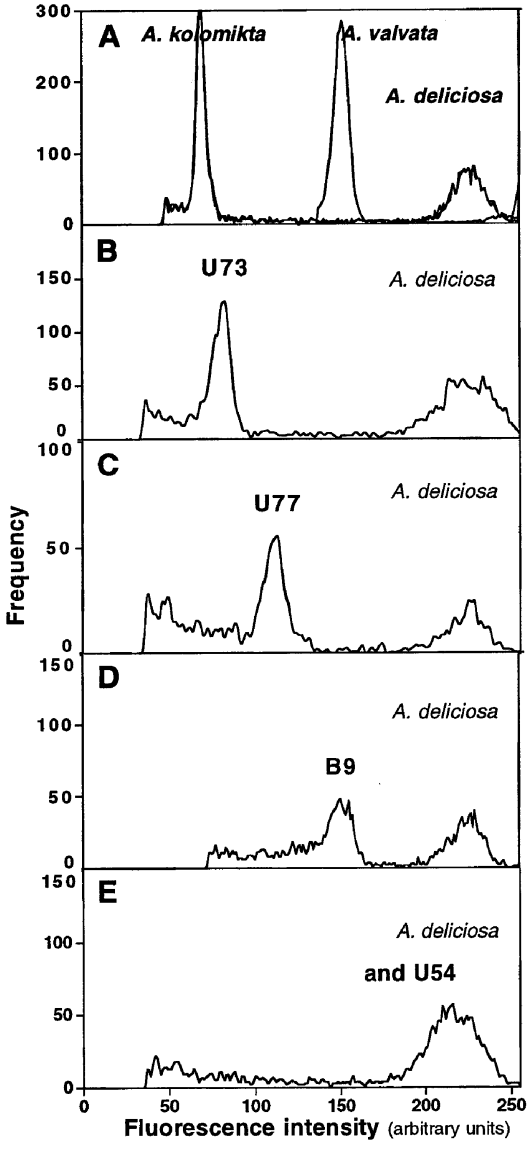

Fig. 1A–E DNA histograms of *Actinidia* species of different ploidy levels and of the progeny of crosses. The histograms are typical measurements of the fluorescent intensity of suspensions of nuclei isolated from leaves of *Actinidia* spp. and stained with bisbenzimide Hoechst 33342. **A** Three contrasting parental species: *A. kolomikta* (2x) cut with *A. deliciosa* (6x) and an overlay of *A. valvata* (4x). **B–E** Progeny recovered by embryo rescue, relative to *A. deliciosa* chopped as an internal standard. **B** Plantlet from the cross U73 [*A. hemsleyana* (2x) × *A. eriantha* (2x)], deduced from the DNA index to be a hyper-diploid; **C** plantlet from the cross U77 [*A. polygama* (2x) × *A. valvata* (4x)], apparently triploid; **D** plantlet from the cross B9 [*A. kolomikta* (2x) × *A. deliciosa* (6x)], apparently tetraploid; **E** plantlet from the cross U54 [*A. arguta* Issa (6x) × *A. deliciosa* (6x)], hexaploid with a DNA distribution contiguous with *A. deliciosa*

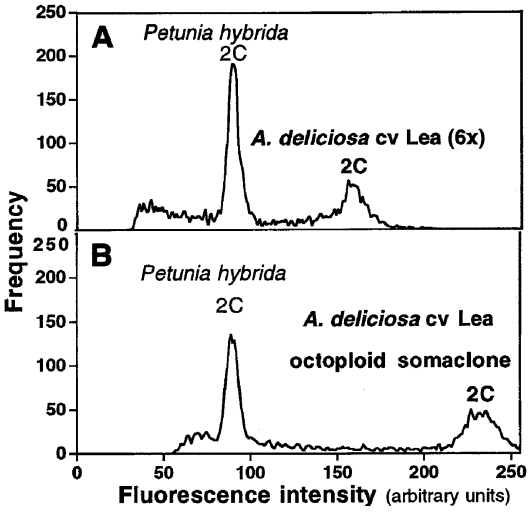

Fig. 2A, B DNA histograms of *Actinidia* cv. Lea and of somaclonal octoploid variants obtained by the vitroculture of young embryos. The histograms depict the fluorescence intensity of Hoechst-stained suspensions of nuclei isolated from leaves of *A. deliciosa* cv. Lea (inconstant male), with *Petunia hybrida* cv. PxPP6 as an internal standard. To allow for specimens with a higher ploidy, we set the photomultiplier gain to a level lower than that in the experiment of Fig. 1. This cultivar is typically hexaploid, as shown in the normal histogram **A**. However, some ploidy variation is readily obtained during the vitroculture of young embryos (at globular and heart stages), as exemplified by the octoploid regenerant giving histogram **B**

shows DNA histograms corresponding to diploid (*A. kolomikta*), tetraploid (*A. valvata*) and hexaploid (*A. deliciosa*) species (histogram A). The progeny of different crosses resulting in diploid, triploid, tetraploid and hexaploid plantlets is represented in the histograms B–E (Fig. 1B–E).

In Fig. 2, histogram A shows the inconstant male (fruiting male) *A. deliciosa* cv. Lea as typically hexaploid, with its octoploid variant (histogram B) obtained from budding calli produced by vitroculture of young embryos. Such changes in the ploidy level express the somaclonal variability easily induced by the vitroculture of juvenile tissue such as endosperm or embryo tissues: nonaploid plantlets (9x) were previously obtained in our laboratory and studied by Ollitraut-Sammarcelli et al. (1994). More recently, Boase and Hopping (1994) detected DNA dodecaploid plants among somaclones obtained by adventitious shoot cultures of *A. deliciosa*.

To summarize, we carried out our breeding program for 4 subsequent years and with 13 different *Actinidia* species. The embryo rescue technique was applied to embryos that had reached the torpedo stage and was the unique tool that ensured the survival of the embryos in four crosses and was also useful to estimate the crossing ability of each species. The earlier investigations of Harvey et al. (1991), Mu et al. (1995) and An et al. (1995) on only a few *Actinidia* species were confirmed.

We designed two sequences of media that enabled the survival of different embryonic stages. The set of nutritive media established for the earliest stages of embryo development did not allow the transition from the globular and heart-shaped stages to the torpedo stage. In an attempt to achieve this, it would be interesting to test supplementing the media with endosperm extracts obtained from the parental species involved in the different crosses, which was not possible in the present investigation due to the distance between the laboratory and the field and the large number of crosses. It is noteworthy that, despite the fact that the globular stage did not lead directly to plantlets, it was relatively easy to obtain the formation of somaclonal variants with a high ploidy level (2n=8x).

All of the crosses carried in Udine that gave seedlings were grown in pots in a greenhouse until the first flowering. For example, the cross *A. chinensis* × *A. kolomikta* (U35), which only succeeded by embryo rescue, flowered in 2000.

The analysis of DNA ploidy level by flow cytometry is a precise and rapid method for studying the parental ploidy level as well as the ploidy level of the progeny that issue from interspecific crosses. The ploidy estimations were made on the leaves or roots of young plantlets. For those species of which the leaves are very rich in polyphenols and mucilages, it is more convenient and more precise to do the flow cytometry estimation on roots.

We found a wide interspecific crossability in *Actinidia* that apparently involved distantly related species belonging to different sections as determined by traditional taxonomic methods. This is an interesting development for breeding, on the one hand, but raises doubts on the consistency of the systematics of the genus *Actinidia* on the other hand. It is worth pointing out that all of the crosses discussed in this paper are controlled crosses and, as such, the competition between pollens of different species is suppressed; this is not the case in open-pollinated crosses.

Breeders should take advantage of the possibility of introgressing characters not present in *A. arguta* and/or *A. deliciosa*, the complex commercially known as kiwifruit, from wild species. The main drawback is the chance of different ploidy levels, which could occur in the pair of species chosen for the cross. This could be a severe obstacle – a wide range of ploidy level has been found in the genus *Actinidia* (Ferguson et al. 1997; McNeilage and Considine 1989) – but such a constraint can be overcome in some instances by a better screening of genotypes within a given species, as the occurrence of ploidy races within the same species has been shown for a number of taxa in *Actinidia* (Yan et al. 1994; Ferguson et al. 1997).

The data that we obtained on crosses between genotypes with different ploidy levels do not support what has sometimes been reported in the literature – notably by Pringle (1986) – that choosing the species with the lower ploidy level as the female parent was more likely to lead to success. In contrast, we observed that unbalanced crosses failed in most instances, independently of the ploidy number of the species used as female parent.

As pointed out above, the wide crossability found in *Actinidia* conflicts with the concept of species, and we probably need to know better just how divergent the *Actinidia* taxa are one from each other. We need to know the relationships between the DNA sequences of the different species (Crowhurst and Gardner 1991). Recently, a study of cytoplasmic genomes was carried out in different *Actinidia* species by means of intraspecific and interspecific crosses. Testolin et al. (1997) revealed a maternal inheritance of mitochondrial DNA and a paternal inheritance of chloroplast DNA in the genus *Actinidia*. The transmission of chloroplast and mitochondrial genomes through different parents provides an exceptional opportunity for studying paternal and maternal lineages of the different species within the *Actinidia* genus. Cipriani et al. (1998) expressed some interesting conclusions concerning the evolution and taxonomy in the genus *Actinidia*. There is a widely reticulate evolution in the genus *Actinidia*, and the systematic classification in the sections and series (Liang 1984) has to be reconsidered due to the fact that the genus *Actinidia* is polyphyletic. This conclusion is strongly supported by the results of our study, which demonstrated the possibility of several successful interspecific crosses between species apparently belonging to different sections.

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