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Full Length Research Paper

Evaluation and potential of Double Immunodifusion Gel Assay for serological characterization of *rice yellow mottle virus* isolates in West Africa

Yacouba Séré[1]*, Amos Onasanya[1], Abolade S. Afolabi[2ψ] and Emmanuel M. Abo[1]

[1]Plant Pathology Unit, WARDA – The Africa Rice Center (WARDA), 01 BP 2031 Cotonou (Bénin)
[2]John Innes Center, Dept. of Crop Genetics, Norwich Research Park, Colney Lane, Norwich NR4 7UH, United Kingdom.

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Rice yellow mottle virus **is not only highly infectious to rice plants but also a highly variable pathogen. Forty-two isolates were obtained from five countries in West Africa. Utilizing 26 polyclonal antisera, the serological diversity of these isolates was determined using Double Immunodifusion Gel Assay. All the antisera were classified into three serogroups, *PSg-1a, PSg-1b* and *PSg-2*. Antisera belonging to *PSg-1a*, *PSg-1b* and *PSg-2* serogroups had diagnostic potential of 86-90%, 69-76% and 52-64%, respectively, for the 42 RYMV isolates analyzed using a dilution of up 1:200. Moreover, all isolates were separated into three serogroups, *Sg-1a*, *Sg-1b* and *Sg-2*. The first two groups are widely distributed across West Africa. The high diagnostic potential exhibited by the 26 RYMV polyclonal antisera indicates that Double Immunodifusion Gel Assay is useful and reliable for diagnosing RYMV. As the use of ELISA (Enzyme-Linked Immunosorbent Assay) is expensive and unavailable in most of the national agricultural research institute in West Africa, they can adopt Double Immunodifusion Gel Assay for the identification and characterization of Rice yellow mottle virus isolates. This is the first phylogenetic analysis report on the use of Double Immunodifusion Gel Assay to characterize Rice yellow mottle virus isolates in West Africa.**

Key words: *Rice yellow mottle virus,* rice, double immunodifusion gel assay, phylogenetic tree, polyclonal antisera, serological diversity, West Africa.

INTRODUCTION

Rice yellow mottle virus (RYMV), genus s*obemovirus* (Hull, 1988), is the most rapidly spreading disease of rice (*Oryza sativa* L.) in Africa (Abo et al., 1998). First identified in Kenya in 1966 (Bakker, 1970), it was reported in West Africa in 1975 (Raymundo and Buddenhagen, 1976). The disease is now found in most African rice-growing countries (Abo et al., 1998). Transmitted by mechanical contact and insects (Abo et al., 1998; Nwilene, 1999), the virus causes yellowing, mottling, necrosis and stunting of rice plants, leading to incomplete emergence of panicles with sterile grains. Severe infection may lead to plant death. Yield losses of 56-68 % have been reported in Niger (Reckhaus and Amadou, 1986), 84-97 % in Sierra Leone (Taylor, 1989), 19-44 % in Burkina Faso (Séré, 1991) and 64-100 % in Mali (Sy et al., 1993). Some farmers have suffered complete crop failure in Côte d'Ivoire (Yoboué, 1989).

The existence of different RYMV strains in the field (N'Guessan et al., 2000; 2001) is often a matter of considerable practical importance. As for most viral diseases, symptoms in the field vary considerably, depending on the rice genotypes, infection strains, stage of infection and environment (Dinant and Lot, 1992). Therefore, reliable criteria are needed for distinguishing and identifying these strains.

Usually, virus isolates are serotyped (Pinner et al., 1988; Rybicki, 1991; Mansour and Baillis, 1994; Konaté

*Corresponding author. Phone: +223 2223375. Fax: +223 2228683. E-mail: y.sere@cgiar.org.
ψCurrent address: 6 Mottram close, Norwich, NR5 8HL, Norwich, UK.

et al. 1997; N'Guessan et al., 2000) using double immunodiffusion gel assay (DIGA) and enzyme-linked immunosorbent assay (ELISA). DIG assay visualizes the antigen–antibody complexes formed after an antigen and antibody have diffused in a semi-solid agar-based medium. The assay offers combinations of antigens and monospecific antisera that have been matched to obtain optimum results using a standard panel of reference antigens. DIG assay is an immunological technique frequently used in plant virus serodiagnosis (Gracia, 2000; Chen, 2002). Although it is increasingly often replaced by more sensitive ELISA techniques, the DIGA is relatively simple, rapid, cheap and practical for detecting virus in few samples (Dinant and Lot, 1992). Therefore, it can be performed easily in any laboratory of the national agricultural research institutes in West Africa.

Little information is available on the use of DIGA for RYMV sero-diversity in West Africa. Consequently, this paper evaluates the potential of Double Immunodifusion Gel Assay for use in serological diagnosis and characterization of RYMV isolates.

MATERIALS AND METHODS

Sample collection

In 1997, 1998, 1999 and 2001, we performed an intensive survey and sample collection in the major agro-ecological zones of Mali, Nigeria, Burkina Faso, Niger and Côte d'Ivoire where rice was produced in upland, lowland and irrigated conditions. Leaf samples were collected on the basis of typical RYMV symptoms. The samples were labeled with the name of location of the collection, stored in an icebox and thereafter transferred to the laboratory and stored in a freezer.

Isolate propagation

The RYMV isolates collected were propagated in the susceptible rice variety Bouaké 189, through mechanical inoculation of 21 days old plants in the screenhouse. Infected leaf samples were ground with 0.01 M phosphate buffer pH 7.0 at the ratio of 1:10 (w/v) and the resulting homogenate filtered through cheesecloth; carborundum powder (600 mesh) was added to the inoculum to aid the penetration of the virus into leaf tissues (Fauquet and Thouvenel, 1977).

Polyclonal antiserum

These antisera were produced as follows: Different RYMV isolates, after propagation on susceptible rice variety Bouaké 189, were purified using the method of Thottappilly and Rossel (1993), which is a modified version of Hull (1988), and Ball et al. (1988). A purified RYMV suspension of 0.5 mg (0.5 ml of 1 mg/ml) was emulsified with 0.5 ml of Freund's complete adjuvant and used to immunize a rabbit; this was followed by three injections with emulsified suspension with Freund incomplete adjuvant. One week after the fourth injection, the rabbit was bled and serum collected. All the 26 antisera produced were pre-adsorbed with healthy plant materials, as described by Pinner and Markham (1990) to remove antibody against host tissue.

Double Immunodifusion Gel Assay (DIGA)

A modified method of Ball (1974) was used. The general procedures were as follow: Leaf samples were ground in 1X phosphate buffered saline-tween-2% polyvinyl pyrrolidone using 1/1, 1/10, 1/20, 1/100,1/200 and 1/500-w/v dilutions. Polyclonal antisera were diluted in saline solution using 1/1, 1/10, 1/20, 1/100, 1/200 and 1/500 v/v dilutions. Ten micro liters (10 µl) of each sample sap were placed in the peripheral wells and 10 µl of the polyclonal antiserum placed in the center agar (0.8% agarose, 0.2% NaN_3, and 0.85% NaCl) wells in Petri dishes. Samples and antisera were applied in three replicates along with negative (maize leaves) and positive controls (leaves from RYMV-infected Bouaké 189 rice cultivar). Petri dishes were incubated at 21.1°C and 25.6°C (room temperature). The first reading and the second one were made under fluorescent light respectively after 5 and 10 days of incubation. After 10 days, test samples showing immunoprecipitin (antigen–antibody complex) bands when compared with positive and negative controls were considered to be RYMV positive (+); those samples without an immunoprecipitin band were considered to be RYMV negative (-).

Phylogenetic analysis

The presence or absence of immunoprecipitin bands was transformed into a binary character matrix ("1" for presence and "0" for absence of a immunoprecipitin band). Pair-wise distance matrices were compiled by using the numerical taxonomy and multivariate analysis system (NTSYS-PC), version 2.1 (Rohlf, 2000) and the Jaccard coefficient of similarity (Jaccard, 1908). Serological diversity dendrogram for 42 RYMV isolates and 26 polyclonal antisera were created by UPGMA cluster analysis (Sneath and Sokal, 1973; Swofford and Olsen, 1990).

RESULTS

The 42 isolates used in the current study are described in the Table 1. The isolates were collected in four agroecological zones: Humid forest, Savanna, Soudano-savanna and Sahel. The 26 polyclonal antibodies raised against RYMV isolates previously collected from different parts of West Africa are listed in Table 2.

Considerable serological diversity was displayed by all the RYMV antisera from different West African countries (Figure 1). According to the pair-wise genetic distances among the antisera analysed at 100% similarity level, all the antisera were completely different with the exception of: (i) RYMV-CI-1 and RYMV-CI-2, (ii) RYMV-M-6 and RYMV-M-7, and (iii) RYMV-M-1, RYMV-M-3, RYMV-M-5, RYMV-BF-2, RYMV-Ng-4 and RYMV-CI-4, each group of which proved to be identical. The first two identical groups were raised from isolates collected in the same countries while the third one include antisera produced from isolates collected in four countries.

At 80% Jaccard similarity level, all the antisera were

Table 1. Identity of isolates of Rice yellow mottle virus collected from rice (*Oryza sativa*) in five West African countries.

S/N	Isolate Code	Country of origin	Locality	Year Collected	Ecosystem	Ecological Zones*
1	MML-001	Mali	Molodo	1998	Irrigated	SH
2	MNN-008	Mali	Niono-8	1997	Irrigated	SH
3	MNN-004	Mali	Niono-4	1997	Irrigated	SH
4	MBG-004	Mali	Baguineda	1998	Irrigated	SO/SA
5	MLG-005	Mali	Longorola(Research Station)	1998	Irrigated	SO/SA
6	MKM-006	Mali	Kayo(Macina)	1998	Irrigated	SH
7	MKG-006	Mali	Kogoni-K6	1998	Irrigated	SH
8	MKG-007	Mali	Kogoni-K7	1998	Irrigated	SH
9	MSL-009	Mali	Selingue	1999	Irrigated	SO/SA
10	MMP-010	Mali	M'Peniesso	1997	Irrigated	SO/SA
11	MKL-011	Mali	Klela	1998	Irrigated	SO/SA
12	BBZ-003	Burkina Faso	Banzon-3	1997	Irrigated	SO/SA
13	BBZ-004	Burkina Faso	Banzon-4	1997	Irrigated	SO/SA
14	BKF-014	Burkina Faso	Karfiguela	1999	Irrigated	SO/SA
15	BBF-015	Burkina Faso	Banfora	1997	Irrigated	SO/SA
16	BBZ-016	Burkina Faso	Banzon	1998	Irrigated	SO/SA
17	NBF-017	Niger	Bonfeba	1997	Irrigated	SH
18	NKK-018	Niger	Kirkissaye	1998	Irrigated	SH
19	NSY-001	Niger	Say-1	1997	Irrigated	SH
20	NSY-002	Niger	Say-2	1998	Irrigated	SH
21	NKL-021	Niger	Kollo	1997	Irrigated	SH
22	NDM-022	Niger	Diomana	1998	Irrigated	SH
23	CTP-023	Cote D'Ivoire	Tepaguia	1997	Lowland	FO
24	COD-024	Cote D'Ivoire	Odienne	1998	Lowland	SA
25	CGH-025	Cote D'Ivoire	Guehiebli	1997	Irrigated	FO
26	CMB-026	Cote D'Ivoire	M'be	1998	Irrigated	SA
27	CSK-027	Cote D'Ivoire	Sakassou	1997	Lowland	SA
28	CDN-028	Cote D'Ivoire	Danane	1997	Irrigated	FO
29	CGL-000	Cote D'Ivoire	Gagnoa-lowland	1998	Lowland	FO
30	CSS-030	Cote D'Ivoire	Sassandra	1997	Lowland	FO
31	CGU-000	Cote D'Ivoire	Gagnoa-upland	1998	Upland	FO
32	NGE-032	Nigeria	Edozigi	1999	Irrigated	FO
33	CKR-001-I	Cote D'Ivoire	Korhogo	2001	Irrigated	SA
34	CKR-034-I	Cote D'Ivoire	Korhogo	2001	Irrigated	SA
35	CKR-024-I	Cote D'Ivoire	Korhogo	2001	Irrigated	SA
36	CTG-005-L	Cote D'Ivoire	Tengrela	2001	Lowland	SA
37	CBD-005-U	Cote D'Ivoire	Boundiali	2001	Upland	SA
38	CBD-008-U	Cote D'Ivoire	Boundiali	2001	Upland	SA
39	CBF-003-I	Cote D'Ivoire	Bouafle	2001	Irrigated	FO
40	CAD-011-I	Cote D'Ivoire	Adzope	2001	Irrigated	FO
41	CTL-017-I	Cote D'Ivoire	Toulepleu	2001	Irrigated	FO
42	CAB-016-L	Cote D'Ivoire	Abengourou	2001	Lowland	FO

* FO = Humid Forest, SA = Savanna, SO/SA = Soudano-savanna, SH = Sahel

Table 2. List of antisera code and origin of samples used to develop them.

S/N	Antiserum Code	Region	Country
1	RYMV-M-1	Niono4	Mali
2	RYMV-M-2	Niono8	Mali
3	RYMV-M-3	M'Peniesso	Mali
4	RYMV-M-4	Molodo	Mali
5	RYMV-M-5	Longorola	Mali
6	RYMV-M-6	Kayo macina	Mali
7	RYMV-M-7	Selingue	Mali
8	RYMV-M-8	Kogoni K7	Mali
9	RYMV-BF-1	Banzon	Burkina Faso
10	RYMV-BF-2	Kafirguela	Burkina Faso
11	RYMV-IITA	IITA	Nigeria
12	RYMV-Ng-1	Saga	Niger
13	RYMV-Ng-2	Kollo	Niger
14	RYMV-Ng-3	Kirkissaye	Niger
15	RYMV-Ng-4	Bonfeba	Niger
16	RYMV-Ng-5	Sayl	Niger
17	RYMV-Ng-6	Diomana	Niger
18	RYMV-CI-1	M'be	Côte d'Ivoire
19	RYMV-CI-2	Danane	Côte d'Ivoire
20	RYMV-CI-3	Gagnoa -L	Côte d'Ivoire
21	RYMV-CI-4	Gagnoa-U	Côte d'Ivoire
22	RYMV-CI-5	Guehiebli	Côte d'Ivoire
23	RYMV-CI-6	Tapeguia	Côte d'Ivoire
24	RYMV-CI-7	Odienne	Côte d'Ivoire
25	RYMV-CI-8	Sakassou	Côte d'Ivoire
26	RYMV-CI-9	Sassandra	Côte d'Ivoire

separated into two major serogroups (*PSg-1* and *PSg-2*), while at 85% *PSg-1* was further separated into two subgroups, *PSg-1a* and *PSg-1b* (Figure 1). According to the composition of each group, some antisera raised from isolates collected in different West African countries appeared to be similar. Conversely, polyclonal produced from isolates collected in a same country may be different.

Antisera belonging to *PSg-1a*, *PSg-1b* and *PSg-2* serogroups (in dilutions of up to 1:200) had diagnostic potential of 86-90%, 69-76% and 52-64%, respectively, for all the 42 RYMV isolates analysed (Tables 3 and 4).

Phylogenetic analysis revealed serological differences among virus isolates (Figure 2). At 50% Jaccard similarity level, all the isolates were separated into two main serogroups (*Sg-1* and *Sg-2*), while at 75%, *Sg-1* was further separated into two subgroups, *Sg-1a* and *Sg-1b*. At 100% similarity level we found that out of the 42 isolates analyzed, (i) 20 isolates were completely different, (ii) 4 from Côte d'Ivoire were similar and (iii) 18 others from each of the five countries involved were also identical.

The distribution of the serogroup across the West Africa ecological zones is indicated in table 5. More or less the same number of samples was collected in each zone. Two seropgroups were found in both the humid forest and soudano-savanna zones while the three serogroups were found in the other zones.

DISCUSSION

Historically, RYMV has been described as a variable virus with many pathological variants (Thottappilly and Rossel, 1993; Konaté et al., 1997; WARDA, 1999; 2001; N'Guessan et al., 2000). The limited number of pathological and morphological characters of RYMV, the inherent plasticity of individual isolates, and lack of standardization of pathological conditions and virulence tests among different researchers has led to confusion and uncertainty in the characterization of this rice pathogen (Mansour and Baillis, 1994; Konaté et al., 1997; Taylor et al., 1990; WARDA, 2001). Distinct pathotypes and virulence phenotypes usually consist of isolates that

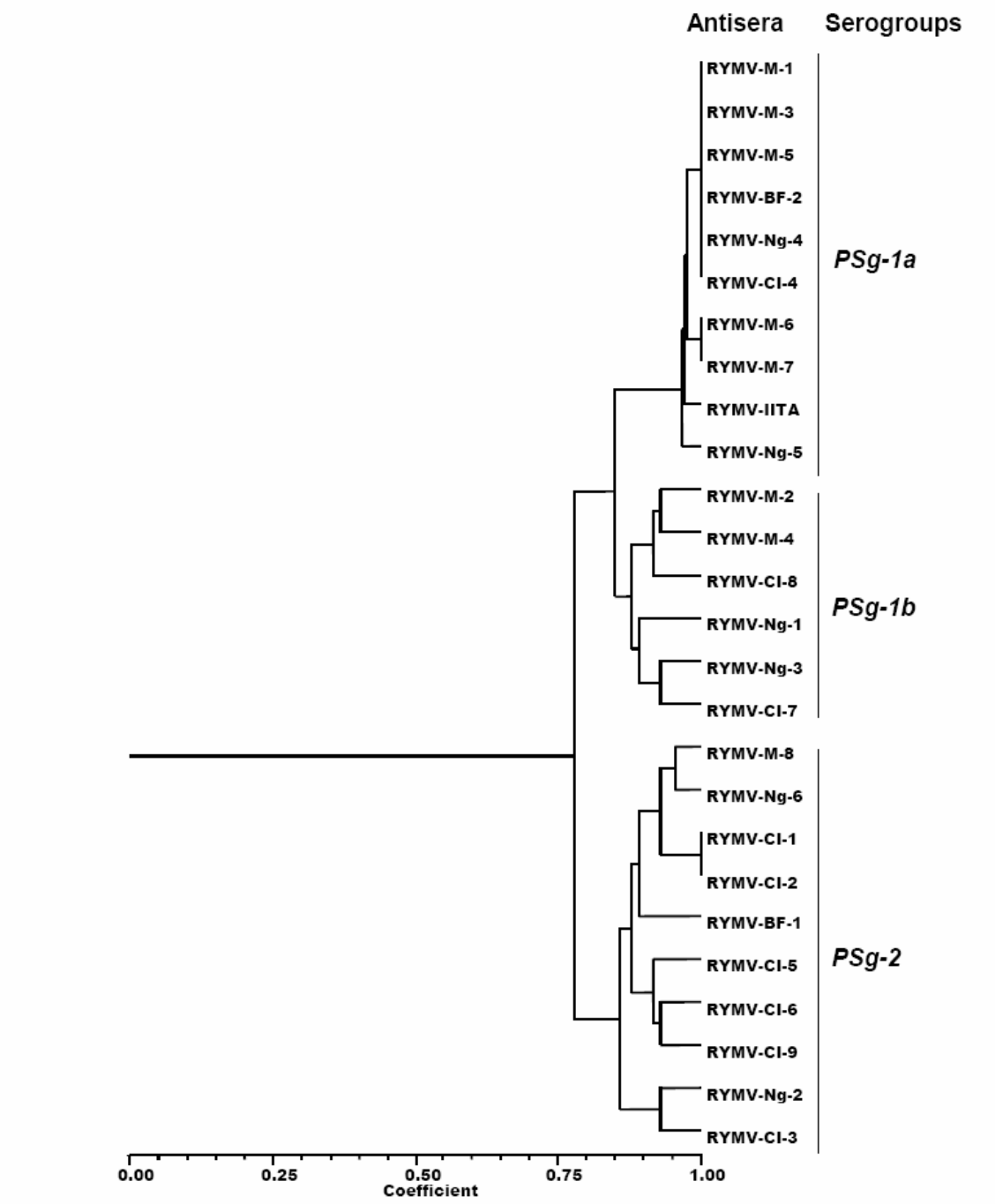

Figure 1. Dendrogram showing serological diversity of 26 West African RYMV polyclonal antibodies.

are more or less serologically unrelated (John et al., 1985; Konaté et al., 1997; N'Guessan et al., 2000), such that identification of strains often lacks consistency and precision (Bakker, 1974; Matthews, 1991). In our study, two isolates (COD-024 from Odiene, and CDN-028 from Danane) that belong to *Sg-1a* and *Sg-1b* respectively, presented the same pathological pattern as they was virulent on a set of eight differential rice cultivars used to differentiate RYMV strains (WARDA, 1999). Conversely, the isolates from Odiene (COD-24) and from Gagnoa (CGL-000) that developed a quite different virulence pattern (WARDA, 1999) belong to the same subgroup (Sg-1a)

The classification of virus isolates into serogroups indicates that several serotypes co-exist despite similarity in the ecological, pathological and degree of virulence of these isolates (Konaté et al., 1997; N'Guessan et al., 2000). In our study, isolates emanating from same

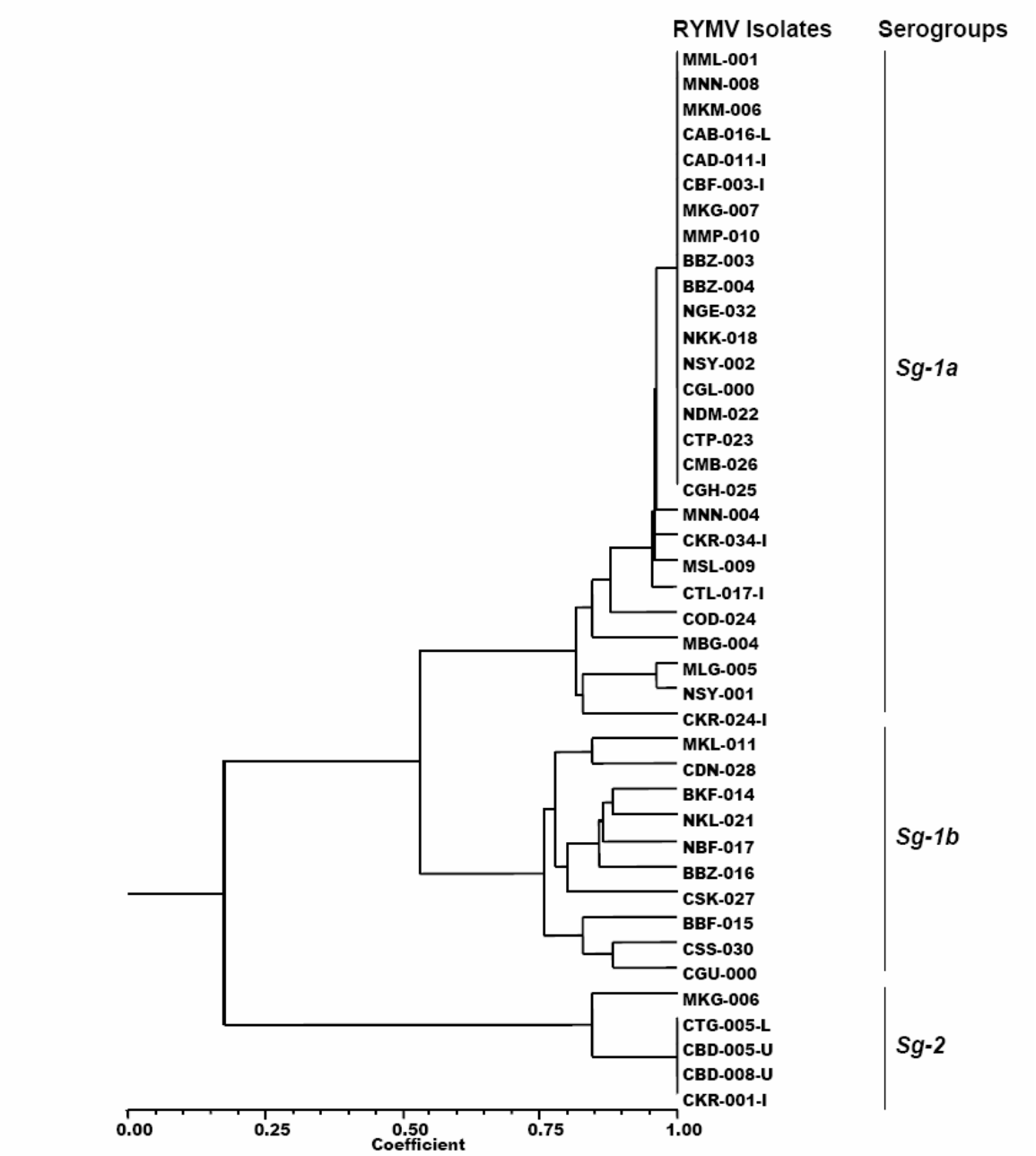

Figure 2. Dendrogram showing serological diversity of 42 West African RYMV isolates.

Table 3. Diagnostic potential of DIG assay using 26 RYMV antisera against 42 RYMV isolates.

Antigen Dilutions (w/v)	Antisera Dilutions (v/v) *					
	1	1/10	1/20	1/100	1/200	1/500
1	++++	+++	++	++	+	-
1/10	+++	+++	+	+	+	-
1/20	+	+	+	+	+	-
1/100	-	-	-	-	-	-
1/200	-	-	-	-	-	-
1/500	-	-	-	-	-	-

* Intensity of immunoprecipitin band: ++++ =very high, +++ = high, ++ = medium, + = low, - = no immunoprecipitin band.

Table 4. Percentage diagnostic potential and diversity of 26 RYMV polyclonal antisera

Antisera	% Diagnostic potential	Serogroup
RYMV-M-1 RYMV-M-3 RYMV-M-5 RYMV-BF-2 RYMV-Ng-4 RYMV-CI-4 RYMV-M-6 RYMV-M-7 RYMV-IITA RYMV-Ng-5	86-90	*PSg-1a*
RYMV-M-2 RYMV-M-4 RYMV-CI-8 RYMV-Ng-1 RYMV-Ng-3 RYMV-CI-7	69-76	*PSg-1b*
RYMV-M-8 RYMV-Ng-6 RYMV-CI-1 RYMV-CI-2 RYMV-BF-1 RYMV-CI-5 RYMV-CI-6 RYMV-CI-9 RYMV-Ng-2 RYMV-CI-3	52-64	*PSg-2*

Table 5. Distribution of the serogroups across the West African ecological zones.

Serogroups	Number by ecological zone				Total by serogroup
	Humid forest	Savanna	Soudano-savanna	Sahel	
Sg-1a	8	4	6	9	27
Sg-1b	3	1	4	2	10
Sg-2	0	4	0	1	5
Total by zone	11	9	10	12	

locality, field and host plant (CKR-034-I and CKR-001-I) were serologically different (Figure 2); this explains the fact that within a set of isolates of related strains, many possibilities of interaction exist (Matthews, 1991). This is also an indication of micro-geographical variation in RYMV population as stated by WARDA (2001), which is practically important for RYMV identification as well as for the deployment of resistant cultivars.

As a result of possible interaction between different strains of the same isolate in the same host plant, which varies between one ecological zone to the other, diverse serological variability tends to exist between different isolates of RYMV in West Africa. The serological similarities observed between isolates within the same and from different West African ecological zones confirm the great cross-infection potential of RYMV transmitted

under natural conditions by different insect vectors (Bakker, 1971; Hammond et al., 1999).

Our study indicated that all the isolates from Côte d'Ivoire forest zone seemed to be serologically related as they belong to *Sg-1* (sub-groups *Sg-1a* and *Sg-1b*), while *Sg-2* group is composed by isolates from the savanna zone. The relative homogeneity of forest zone isolates was confirmed using five discriminating monoclonal antibodies in ELISA test, indicating that all the isolates of Cote d'Ivoire forest zone were into the same group (WARDA, 1999). In a study on serological variability of RYMV isolates, N'Guessan et al. (2000) found two distinct serotypes (S1 and S2). The S2 was more prevalent in Cote d'Ivoire than S1 and was found mainly in the central and southern part of the country. Conversely, S1 occurred more widely in the North and in the neighboring countries of the North of Cote d'Ivoire. Our study supports also a different distribution pattern of the serogroups according to their agro-ecological zone of origin. The two sub-groups *Sg-1a and Sg-1b* were widely distributed in West Africa as they were found in the four agro-ecological zones and in the five countries. Conversely, isolates belonging to *Sg-2* serogroup have the smallest pattern of distribution in West Africa**.** The serotype *Sg-1b* might suggest many possible interactions and co-existence between *Sg-1a* and *Sg-2* serotypes. The successive transmission by beetles and other insects of such mixtures with *Sg-1a* prevalence may progressively have lead to elimination of *Sg-2* (Urban et al., 1990). This further explains the prevalence and wide distribution of *Sg-1a* serotype in West Africa. It was hypothesized that such possibilities of interaction within a set of isolates of related strains might lead to frequent occurrence of mutants, which might be responsible for the high level of serological variation among the isolates (Boccard and Baulcombe, 1993). More research involving the use of molecular techniques is needed to confirm if further sub-groupings would be appropriate, since there was a good correspondence between the serological and molecular diversity of RYMV isolates (N'Guessan et al., 2000).

The observed low antisera diagnostic potential of 52–64% (*PSg-2* serogroup) against all virus isolates tested may indicate that virus exists in the wild and grass as a population from which different strains may emerge to cause disease in rice (N'Guessan et al., 2000). The high diagnostic potential exhibited by the 26 RYMV polyclonal antisera is highly suggestive of their usefulness and reliability in the diagnosis of RYMV disease in West African rice agro-ecological zones. This would, undoubtedly, facilitate easy and early diagnosis of the disease in order to develop control measures based on sanitation.

Our results demonstrate that the serological data generated by DIGA is reliable and possess great potential for assessing serological diversity of RYMV isolates and polyclonal antibody characterization in West Africa. It was able to reveal the existence of serological diversity among virus isolates within and among West Africa countrie. To our knowledge, this is the first phylogenetic analysis study on the use of DIGA to serotype and characterize RYMV isolates. The knowledge of virus sero-diversity using this technique should be useful for the inspection of RYMV in rice-growing regions, as well as assist breeding programmes aiming at the effective development of cultivars with durable resistant to RYMV.

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